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INNATE IMMUNE AIRWAY RESPONSES AFTER EXPOSURE TO ULTRAFINE AND AMBIENT PARTICLES: IN VIVO AND IN VITRO MODELS

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Innate Immune Airway Responses after Exposure to Ultrafine and Ambient Particles:
In vivo and *in vitro* models

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Till nära och kära

ABSTRACT

Inhalation of ultrafine and ambient particles in the air triggers a response in the innate immune system of the airways. This thesis explores measures to reduce exposure to organic dust, to dampen the adverse immune effects of chronic organic swine dust exposure and develop a refined *in vitro* bronchial mucosa model to reduce *in vivo* toxicity testing on humans and animals.

In Paper I, the aim was to reduce particulate matter exposure by installing particle separators in swine buildings and to explore the respiratory effects in healthy subjects after acute exposure. Exposure measurements including organic dust including endotoxins in both swine building environments (with and without particle separation) were performed and the particle separators reduced mainly particles sized 0.3-0.5 µm. The adverse acute symptoms like headache and increased body temperature in the volunteers were reduced when exposed to the particle separated swine building environment compared to the conventional swine building environment. The particle separators reduced the pro-inflammatory responses (IL-6 and CXCL8) in the upper respiratory tract compared to the conventional swine building environment.

In Paper II, the aim was to investigate the host innate immune response *in vivo* in chronically organic dust exposed swine farmers after short-term glucocorticosteriods therapy. Swine farmers inhaled budesonide for two weeks which increased their release of soluble TLR2 in the airways. Systemic effects included increased number of circulating leucocytes and TLR4 expression on lymphocytes, and decreased cytotoxic T-cell production of IL-13 and IL-4.

The second aim of Paper II was to elucidate the cellular immune response of alveolar macrophages from chronically exposed swine farmers to *ex vivo* co-stimulation of glucocorticosteroids and TLR ligands. In alveolar macrophages, mRNA *TLR2* expression increased and *CXCL8* decreased after *ex vivo* co-stimulation with LPS/peptidoglycan/TNF-α and budesonide. The mRNA expression of *CD14*, *IL-13* and *GPx* in alveolar macrophages increased after the *in vivo* steroid treatment of swine farmers. In all, this study showed that inhalation of a glucocorticosteroid strengthens the immune defense pathways in subjects with occupational chronic exposure to organic dust.

In Paper III, the aim was to develop an organotypic *in vitro* exposure system; combining bronchial models with Xpose*ALI*® for exposure of nano-sized palladium. Here we established a viable and robust *in vitro* bronchial mucosa co-culture model using human primary bronchial epithelial cells and a fibroblast cell line showing *in vivo* characteristics. By stimulation with IL-13, the model differentiated into a chronic bronchitis-like model. It was successfully combined with the advanced aerosol exposure system PreciseInhale™ and the *in vitro* module Xpose*ALI*® and exposed to palladium nanoparticles, which induced inflammatory responses in the 3D models.

LIST OF SCIENTIFIC PAPERS

I. **A Hedelin***, B.M. Sundblad, K. Sahlander, K. Wilkinson, G. Seisenbaeva, V. Kessler, K. Larsson, L. Palmberg
Comparing human respiratory adverse effects after acute exposure to particulate matter in conventional and particle-reduced swine building environments
Occupational and Environmental Medicine 2016, 73 (10): 648-655

II. **A. Steneholm**, B.M. Sundblad, S. Kullberg, J. Grünewald, K. Larsson, L. Palmberg
Effects of inhaled steroids on innate immunity in swine farmers; A cross-over study
Manuscript

III. J. Ji, **A. Hedelin***, M. Malmlof, V. Kessler, G. Seisenbaeva, P. Gerde, L. Palmberg
Development of combining of human bronchial mucosa models with XposeALI® for exposure of air pollution nanoparticles
Plos One. 2017, 12 (1): 1-17

* Hedelin was the former last name of Anna Steneholm

CONTENTS

LIST OF ABBREVIATIONS

ALI	Air-liquid interface
AM	Alveolar macrophages
APC	Allophycocyanin
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BM/AM	Basal/apical lavage medium
Bud	Budesonide
CD	Cluster of differentiation
CE	Conventional swine building environment
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
DAMP	Danger-associated molecular pattern
ELISA	Enzyme linked immunosorbent assay
FEV_1	Forced expiratory volume in one second
FITC	Fluorocein isothiocyanate
FVC	Forced vital capacity
GPx	Glutathione peroxidase
ICS	Inhaled corticosteroids
IFN	Interferon
IL	Interleukin
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccaride
MUC5AC	Mucin 5AC
MyD88	Myeloid differentiation primary response protein 88
NO	Nitric oxide
ODTS	Organic dust toxic syndrome
PAMP	Pathogen-associated molecular pattern
PBEC	Primary bronchial epithelial cells
PCR	Polymeras chain reaction
PE	Phycoerythrin

PEF	Peak expiratory flow
PerCP	Peridinin chlorophyll protein
PM	Particulate matter
PRR	Pattern recognition receptor
PSE	Particle separated swine building environment
sCD14	Soluble cluster of differentiation 14
sST2	Soluble suppression of tumorgenicity 2
SEM	Scanning electron microscope
sTLR	Soluble toll-like receptor
T_c	T-cytotoxic cell
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscope
T_h	T-helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
VC	Vital Capacity

INTRODUCTION

Particulate matter - particles that matter

Or do they?

In this thesis we are trying to shed light on how ultrafine and ambient particles, matter to humans.

Inhalation of particles affect the human immune system. But is it necessarily bad? Are there adverse effect?

Some people are allergic to agents they inhale; like pollen, house mite dust or dander from cat and dog. These cases are generally easy to spot, diagnose, and even treat. But the particles that don't cause these acute, obvious effects – are they also hazardous to human health? Yes and no. There are many factors that need to be considered. It is not only the composition of the particle itself, but also the size, shape and dose that matters. Does inhalation of particles have any immunological effects in the human respiratory tract and how are these effects measured? Elucidation of these questions is the reason why you should continue reading.

PARTICLES

Characteristics and hazard

Particulate matter (PM) air pollution is an important risk factor for adverse health effects. It consists of agglomerates of solid and liquid particles suspended in air and vary in origin and composition. Size is an important parameter to understand the fate for settling on surfaces and fate primarily in the respiratory system but also the rest of the body. The largest measured PM fraction, identified as PM_{10} are coarse particles with a median aerodynamic diameter (MAD) $\leq$10 µm. PM_4 is often used as a cut-off for respirable particles, however it is the fraction $PM_{2.5}$ (MAD$\leq$2.5 µm) that has and continues to get increased regulatory attention.

WHO global air quality guideline limits for $PM_{2.5}$ and PM_{10} are: 10 µg/m^3 for the annual average (25 µg/m^3 for the 24-hour mean, not to be exceeded for more than 3 days/year) and 20 µg/m^3 for the annual average (50 µg/m^3 for the 24-hour mean), respectively [1].

Recent advances in nanotechnology has led to an increase in the manufacture and use of nanoparticles which are engineered to be <100 nm in one dimension. The unique properties has enabled them to be used in a variety of industrial and consumer products, such as sun-creams, paints, clothes, cosmetics and spray cleaners. Due to their use in consumer products and media coverage, the public have become more aware of **nanoparticles** and the possible presence in their daily life. To better understand the volumes in use registration of nanoparticles is currently mandatory in Belgium, France, Denmark, Norway and Sweden. Also in the environment presence of micro- and nanoplastics are an emerging topic of concern. The interest of nanoparticles from authorities and researchers is linked to scarce knowledge of human exposure and toxicity of these small particles. Yet, human exposure to small airborne particles

is not new. Bacteria and viruses, forest fires, volcanic activities and strong winds over sea or dessert are all natural contributors to ambient particles called **ultrafine particles** (<100 nm). Not only natural, but also anthropogenic sources such as combustion engines, frying and grilling of food and welding fumes contribute to human inhalation of ultrafine particles [2]. Notably, size-wise the diameter of the ultrafine particles is more than 100 times smaller than the diameter of a PM_{10} particle.

Biological effects of particle exposure

The possible **hazards** of nano- and ultrafine particles are often linked to their large and reactive surface area [3]. For understanding the toxicity including the fate of the particles in air and the respiratory tract, physical characterization of the particles is essential. Their ability to form agglomerates from primary particles, into larger ultrafine to microparticles, secondary particles influences the hazard potential.

Exposure to ambient particulate matter and its association to cardiovascular morbidity/ mortality is well-established [4, 5] likely due to the induced systemic inflammatory response [6, 7]. The exposure also correlated to the hospital admissions of asthma and COPD [8, 9]. The World Health Organization IARC reviewed existing carcinogenicity data and classified both outdoor air pollution and particulate matter in outdoor air pollution as carcinogenic to humans due to sufficient evidence in humans. The group claims that the agents "are associated with increases in genetic damage that have been shown to be predictive of cancer in humans. Moreover, exposure to outdoor air pollution can promote cancer progression via oxidative stress, responses to oxidative stress, and sustained inflammation" [10].

Occupational exposure

Occupational exposure to particles and dust in general have long focused on quantity rather than quality. Occupational hygienist have measured the total inhalable dust sized <100 µm, which is the fraction of airborne materials that enters the nose and mouth during breathing or respirable dust which is the fraction sized <10 µm, that could reach the small airways and alveoli where the gas exchange takes place. With increased focus on reducing the occupational exposure to dust combined with improved analytical techniques and advances in inhalation toxicology it has become evident that the size of particles is of importance. For instance, recently the Swedish occupational exposure limit of 5 mg/m^3 for organic dust changed from "total dust" to "inhalable dust" [11].

Known occupational exposure to nano- and ultrafine sized dust and particles occurs in groups exposed to ambient particles like traffic police and occupational exposure in welders and farmers.

In general, measuring occupational exposure to bioaerosols or organic dust, which contain a heterogeneous mixture of agents is challenging [12]. In order to evaluate trends across studies, it is important to compare the personal occupational exposure data of the subjects. This is often challenging, as crucial information of sampler, airflow of sampler, monitoring duration and

tasks that were monitored might be lacking in the reports. This is especially true when evaluating endotoxin monitoring and analysis (see Methods).

Dust in swine farms is characterized by its complex composition of mainly organic material. In Swedish swine farms, the total inhalable dust has been detected at 10 mg/m^3 and respirable dust 0.3 mg/m^3 [13] but could be higher (up to 28.5 mg/m^3) depending on the level of activity of the workers and the pigs. In a large Danish study where exposure in 53 farms was investigated, the mean exposure for inhalable dust was 3.4 mg/m^3 [14]. It was observed that the levels of organic dust were significantly higher in winter than summer which could be explained by less use of the ventilation in the winter to save heat and energy in the stables. Other important activities known to increase the level of exposure to swine dust is high-pressure water cleaning of stables and weighing (or other manual handling) of pigs [15]. The organic swine dust originates from feces, feed, dander, and contains numerous constituents like pro-inflammatory endotoxins and peptidoglycan. **Endotoxins**, first described more than 100 years ago, are the heat-stable lipopolysaccarides (LPS) of the outer part of the Gram-negative bacteria. Endotoxin is generally detected in agricultural settings, cotton production and waste processing. Compared to bacteria, endotoxins/LPS are long-lived and can only be inactivated though (dry) heating for several hours [16, 17]. National occupational exposure limits for endotoxin are not established to date. The Dutch Expert Committee on Occupational Safety proposed, based on the acute respiratory effects, a health-based exposure limit of 90 EU/m^3 in 2010, that has not yet been adopted [18].

Nevertheless, dust and endotoxins levels exceed current occupational exposure limits in many animal facilities. In 2012, 93% of the swine, poultry, mink and dairy farms in Denmark did not meet the suggested Dutch endotoxin OEL of 90 EU/m^3. Basinas *et al*, 2012 examined the day-to-day exposure of swine and dairy farmers and the influence of working indoor versus outdoor [19]. Measurements from 124 personal samplers of farm workers of 26 Danish dairy farms showed mean concentrations of dust of 1.0 mg/m^3 dust and endotoxin 360 EU/m^3 and the conclusion was to recommend the use of respirators for certain dust generating work tasks like feed handling and bedding (manure) removal [20]. The same research group later investigated exposure in Danish livestock facilities reported in 41 studies and among the swine, poultry and cattle farms exposure of inhalable dust was 0.8-10.8 mg/m^3 and endotoxin exposure was 400-6600 EU/m^3 [21]. Exposure to inhalable dust and endotoxin in swine and poultry farming was higher than for cattle, but the peak exposures were often linked to a specific task. Even though the highest average exposure to endotoxins were measured for swine farmers, poultry farmers had higher dust and endotoxin exposure during the work in the stables.

Peptidoglycan (PGN) originates from Gram-positive bacteria and is established as an important pathogen-associated molecular pattern (PAMP) recognized by TLR2 [22] and known to cause inflammation in epithelial and alveolar cells [23]. Peptidoglycan has been suggested to be, together with LPS, important in the development of endotoxin tolerance observed among swine farmers exposed to organic dust [24]. *In vitro*, peptidoglycan tolerance has been correlated with induced IRAK-M protein [25] but several mechanisms for the observed tolerance have been suggested without any scientific consensus [26].

To improve occupational conditions and the environment for growing farm animals different **measures to reduce exposure** have been explored [27], some more successful than others [28]. Spraying vegetable oils that form a fog and binds particles was successful, however it is a less commonly employed measure nowadays [29, 30]. The efficiency of oil spraying is decreased as the humidity increases resulting in only minimal reductions in the endotoxin concentration [31]. The use of respiratory protective equipment could be efficient [13, 32, 33], but not to all contaminants in the air, e.g. gases [34]. High-pressure water jet cleaning of the stables is known to be one of the necessary tasks that causes high exposure of swine dust to farmers [35]. Pre-cleaning the stable using a robot reduces the inflammatory response systemically as well as in the upper and lower airways of healthy volunteers [15]. Such measure would likely have a positive health impact in swine farmers, as it would reduce the duration of work in highly contaminated stables. A relatively simple, and still successful, intervention tested in order to lower the exposure was to send a letter including information on previous exposure measurements at the farm accompanied by advice on how to reduce exposure. Thus if farmers were aware they were exposed to high concentrations, they were more likely to use some of the advices in the letter [36].

THE RESPIRATORY SYSTEM

Breathing – the inhalation of oxygen, the exhalation of carbon dioxide and the exchange of gases in the alveoli of the lungs – is crucial for human survival. The inhaled air will pass the nose (and mouth), further change directions from horizontal to vertical during the passage through the pharynx, larynx, and further down to the lower respiratory tract via the trachea, bronchi and bronchioles and finally the alveolar ducts and the alveoli where it then makes the reverse journey during exhalation. In the trachea, bronchi, and bronchioles, cilia cover the airway epithelium, which is covered with mucus, functioning as a mechanical barrier of the body (Figure 1). Mucus produced by goblet cells or submucosal glands traps foreign particles and the synchronized beating cilia transport the mucus towards the pharynx to be swallowed. The mucociliary clearance is an efficient first line of defense of the respiratory system. The second line of defense is the nonspecific innate immune system (see Innate immunity).

The tidal volume contains about 0.5 liter of air. During physical activity, the tidal volume and the breathing frequency increases in order to increase ventilation. A human at rest breathes 10-15 m^3/day whereas an active worker could easily inhale the same amount of air during an 8-hour work shift. Lung capacity is also influenced by age, height, length, weight and ethnicity.

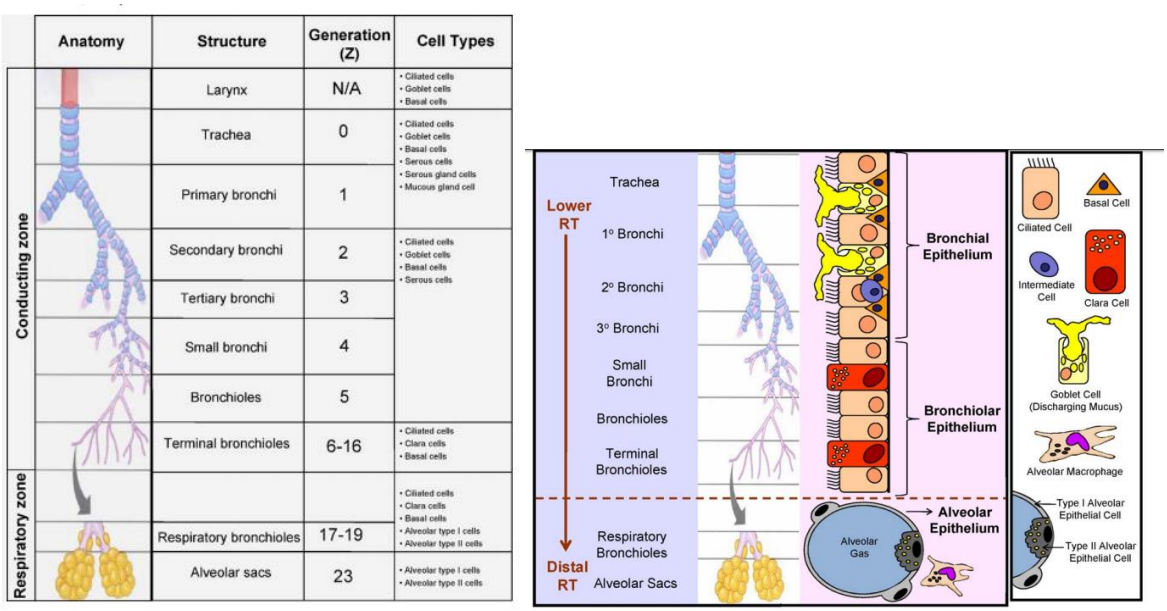


Figure 1. Anatomy and cell types of the respiratory tract [37]

Bronchial epithelial cells line the lower airways and play an important role in protecting the host against inhaled xenobiotics. Several types of cells comprise the human bronchial epithelium: ciliated cells (50-70%), basal cells (30%), mucus producing goblet cells (<25%) and Club cells (1-11%) [38, 39].

Ciliated cells line the airway epithelium from larynx down to the last generation of bronchioles. The coordinated beating of the cilia facilitates transport of the mucus to the pharynx. The ciliary axoneme is characterized by the two central singlet and nine outer doublet microtubules, about 0.25 µm in diameter and can vary in length from a few micrometers to up to 2 mm [40]. Ciliated cells originate from the **basal cells** which are considered stem cells as these could also mature into the other functional epithelial cells [41].

Goblet cells are membrane-bound mucus producing cells. The mucus consists of water, ions, and mucins with the most prominent being mucin 5AC (MUC5AC). Mucus forms a physical barrier in the airways and is important for the mucociliary clearance by trapping inhaled particles. Overproduction of mucus is characteristic for acute and chronic respiratory diseases like bronchitis, asthma and cystic fibrosis [42]. Meta- and hyperplasia of goblet cells is observed in chronic bronchitis and can be induced by IL-4 and IL-13 *in vitro* [43, 44]

Club cells formerly known as Clara cells, are recognized in electron microscope by their electron-dense lamellar bodies. These cells also have stem cell properties. In the lung, the club cells contain the highest concentration of cytochrome P450 oxidase, important for metabolism of xenobiotics. Club cells can produce the pro-inflammatory club cell protein: CC16 (also known as CC10 or uteroglobin). In COPD patients and smokers, CC16 in serum and BALF are significantly lower than in healthy non-smoking individuals [39, 45].

THE IMMUNE SYSTEM

Innate Immune System

The second line of defense of the airways is the nonspecific innate immune system. Consisting of circulating phagocytic macrophages, dendritic cells and neutrophils, it acts together with the structural cells (epithelial cells, Club cells, mast cells and fibroblasts) to provide host defense.

Macrophages in the airways, called alveolar macrophages (AM), have two origins: resident macrophages or monocytes recruited from blood into the lung and differentiated into macrophages. The former have good phagocyting capacities while the monocyte-derived AM have enhanced abilities to initiate an acquired immune response by secreting and responding to a range of effector molecules [5]. Phagocytosis includes several crucial steps required for full functionality, which may be impaired by air pollution and cigarette smoke [46]. Like T-cells, also macrophages can polarize into the pro-inflammatory M1 or anti-inflammatory M2 subtypes. Stimulation with LPS , IFN-γ and TNF-α will activate the classic M1, which is able to resist pathogens and excrete pro-inflammatory cytokines and reactive oxygen and nitrogen intermediates [47]. The polarization into M2 is initiated by IL-4, IL-10 and IL-1 [9, 48]. Diesel exhaust particles and cigarette smoke also activate M2, whereas air pollution PM rather seems to activate M1 [49]. Although AM can produce many cytokines, they likely are not likely the main contributor in the airways of cytokines, especially the late-phase ones which lymphocytes produce [46].

T-cells lymphocytes are part of the adaptive immune system and consist of many subsets of cells including CD4+ T-helper cells and CD8+ cytotoxic cells. Originating from bone marrow, T-cells mature in the thymus and are finally activated and differentiated to T_h or T_c cells in the body. Depending on the activation, the T-cells polarize into pro-inflammatory cytokine releasing T_h1/T_c1 or anti-inflammatory cytokine releasing T_h2/T_c2 cells[50]. The “innate” role of T-cells cannot be dismissed as some subsets, like memory and regulatory T-cells, express TLRs [51-53].

Neutrophils are the most abundant leucocyte in the circulating system and important for the host defense against pathogens. The cell is short-lived (hours-days) but upon migration (infiltration) into inflamed tissue (by chemo-attractants like CXCL8 released by macrophages) it’s life-span is extended [54]. After the cells have eliminated the invaders by phagocytosis they undergo apoptosis [55]. During the phagocytosis, the neutrophil requires additional oxygen which in turn lead to the production of reactive oxygen species (ROS) that in itself could increase the inflammation [56].

The **Pattern Recognition Receptors**, PRRs, is a family of preserved proteins crucial for the innate immune system, but also important for interacting with the adaptive immune cells. The receptors are present in immune cells and both endo- and epithelial cells, located on the cell membrane as well as in the cytoplasm and can bind pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Four families have been identified: toll-like receptors (TLRs), nucleotide binding and oligomerisation domain-like

receptors (or NOD-like receptors (NLRs)), C-type lectin receptors (CLRs) and retinoic acid-inducible gene 1-like receptors (RLRs) [57].

The fruit fly *Drosophilia melanogaster* toll gene and toll receptors were discovered 20 years ago and soon thereafter, the first mammal homologue was consequently named **toll-like receptors** or TLRs [58]. Currently twelve TLRs – ten in humans – have been identified, which each recognize specific PAMPs. Six are expressed on the cell surface: TLR1, TLR2, TLR3, TLR4, TLR5 and TLR6 whereas four are located in intracellular vesicles like endosomes: TLR3, TLR7, TLR8 and TLR9. Many studies have tried to elucidate the ligands of the different TLRs but often the sample preparations were contaminated with LPS or other potent immune-activators, thus more research is required [59].

Important ligand(s) for this thesis is ambient PM and especially organic swine dust which could contains both Gram-positive and -negative bacteria and fungi which are recognized in humans predominantly by TLR2 and TLR4 [9, 60].

TLR2 was discovered 1999 and is an important receptor for peptidoglycans [61], however it is also one of the most non-specific TLRs in terms of ligand binding. It binds exogenous lipoteichoic acid (LTA), lipoproteins and LPS from non-enterobacteria, but also endogenous ligands like heat-shock proteins [59, 62]. The receptor is located on the cell surface and recognizes its ligands by forming heterodimers with TLR1 or TLR6 [57]. To induce the release of pro-inflammatory cytokines and chemokines, TLR2 activates the MyD88-dependent pathway that triggers NF-κB that initiates the inflammatory release of cytokines and chemokines [63].

The soluble form of TLR2, sTLR2, is present in many compartments including human plasma, BALF, sputum, saliva and breast milk [64]. Shedding TLR2 from the plasma membrane of sputum neutrophils into sputum of smokers [65] might be a protective mechanism to reduce the numbers of receptors and thereby the pro-inflammatory host defense.

Farmers´ blood monocytes express less TLR2 compared to smokers and healthy controls [24], whereas data on COPD-patient are ambiguous [66, 67]. TLR2 expression in AM from COPD patients have also been shown to be inconsistent [68, 69]. Smokers have lower *TLR2* AM expression [66] and smoking COPD patients lower expression of TLR2 on sputum neutrophils than non-smoking healthy controls [65].

Endotoxins were mentioned in the end of the 19th century, LPS was discovered in the 50's and the Gram-negative bacteria-induced sepsis has been known since the mid 60's. Yet it was not until the end of the last century that the first human TLR was identified: **TLR4**. Soon it was clear that this was the missing link between adverse human effects and the innate immune system and the discovery was awarded the Nobel prize in 2011 [58]. The main and thoroughly studied TLR4 ligand LPS also called endotoxin is the major component of the outer membrane of Gram-negative bacteria. LPS is shed in small amounts during bacterial growth and binds to the LPS-binding protein (LBP) in plasma. The complex is delivered to the cell-surface receptor

TLR4 that forms a complex with MD2 and CD14. Not only LPS could be part of the TLR4/MD2/CD14/LPB-complex but also endogenous ligands like heat-shock proteins are possible agonists even though they require very high concentrations to activate TLR4 [59]. LPS has high affinity for the receptor and is likely present in swine dust, thus it is unlikely the endogenous ligands are responsible for the very high immunomodula-tory properties. Similarly to sTLR2, a soluble form of TLR4 has been found in saliva and plasma in humans [64, 70].

Although TLRs were discovered at the end of the 90's, **CD14** (a co-receptor of TLR4) had already been discovered in 1990 [71]. It is a LPS-binding receptor bound to the cells surface of monocytes, macrophages and neutrophils but exists also abundant as soluble form (sCD14) in serum, sputum and BALF [65]. After binding of LPS to LPB, either membrane-bound or soluble CD14 presents LPS/LBP to the TLR4/MD2 complex. Thereby, the soluble form is also able to induce LPS-activation in also low membrane-expressing cells like epithelial and endothelial cells [72]. If LPS is present in high concentrations, sCD14 has been shown to relocate CD14 membrane-bound LPS to plasma lipoproteins. CD14 binds not only LPS, but also peptidoglycans, which cannot activate epithelial and endothelial cells like LPS does [73]. Upon CD14-initatied LPS-activation the pro-inflammatory production of cytokines is commenced. As sCD14 in liver cells induce expression of IL-6, sCD14 is considered an acute phase protein [74].

Inflammatory mediators

Interleukin-8 (IL-8) or **CXCL8** is a chemokine produced by many types of cells including macrophages, epithelial cells and endothelial cells and is induced by pro-inflammatory cytokines, viruses and bacteria including LPS. Major biological activities include promoting adhesion of monocytes and fibroblasts as well as functioning as a chemoattractant allowing neutrophils to infiltrate into the airways, causing respiratory burst and inducing MMP-9 release [75].

Interleukin-6 or **IL-6** is a pro-inflammatory cytokine produced by immune cells like alveolar macrophages, monocytes and neutrophils but also epithelial cells and fibroblasts. The release of IL-6 can be induced by cell stress or damage caused by UV, ROS, LPS, viruses etc. Via the bloodstream, IL-6 enters the liver where it induces acute phase proteins like C-reactive protein (CRP) [76]. The concentration of CRP is often measured in primary care units as a biomarker for acute or ongoing inflammation caused by bacteria rather than virus. Although the cytokine IL-6 itself likely acts by multiple mechanism and is important for the pathogenesis of respiratory diseases such as asthma and COPD [77].

Soluble suppression of tumorigenicity 2 or **sST2** is one of four isoforms of ST2, which is the target receptor of the newly described IL-1-like cytokine IL-33. The binding of IL-33 to sST2 inhibits the binding to membrane-bound isoform ST2L and thereby acts as a decoy receptor. The ST2L is only expressed in T_h2-cells, mast cells and cardiomyocytes whereas the soluble form is present in almost all living cells. The increased expression levels of sST2 in heart cells as a response to myocardial stress including infarction is well studied. Also in respiratory

inflammation, the IL-33/ST2 activates T_h2 effector cells and influence T_h2 related cytokines like IL-4, IL-5 and IL-13, all important in e.g. asthma and pulmonary fibrosis. Serum levels of sST2 have been shown to be higher in COPD- [78] and asthma patients during exacerbations than in controls [79, 80]. Exposure to LPS and swine dust increase serum sST2 in healthy controls, but not in swine farmers [81].

IL-13 is a regulatory anti-inflammatory cytokine produced primarily by T_h2-cells but to some extent also mast cells, eosinophils and macrophages. Numerous studies show that IL-13 induces mucus hypersecretion and data from animal models indicated that IL-13 is crucial for asthma and COPD development. The IL-13 concentration in blood is higher during exacerbation of asthmatics compared to during non-episodes, which is not found in COPD patients in general [82, 83]. Yet comparing smokers with and without chronic bronchitis, the number of IL-13 expressing cells in bronchial submucosa is higher in the smoker with chronic bronchitis [84].

Matrix metalloproteinase-9 or **MMP-9** belongs to a family of zinc-dependent proteinase secreted constitutively by neutrophils and eosinophils and during inflammation by macrophages and mast cells. MMP-9 degrades proteins like collagen and elastin of the extracellular matrix but can also cleave non-extracellular matrix proteins like chemokines. This degradation is important for tissue remodeling, a distinct feature of inflammation of the airways in diseases like asthma. Upon secretion of metalloproteinase-1 (TIMP-1) to the extracellular space, TIMP-1 can bind and inhibit MMP-9, important for the tissue homeostasis [85]. Steroid-treated asthma patients increase submucosal expression of TIMP-1 and decrease MMP-9 [86]. In COPD patients, the severity of the disease correlates to the serum MMP-9 concentrations [87].

Airway immune system in disease

Chronic bronchitis is defined as daily chronic productive cough for at least three months during two consecutive years. The increased production of mucus is caused by excessive tracheobronchial goblet cell hyper- and metaplasia and increase of the submucosal glands leading to a thickening of the bronchial wall. The pathogenesis is unclear but bacterial colonisation and the resulting inflammatory response seem to be important for the progression of the disease [88]. Of all diagnosed patients, 90% are smokers or ex-smokers [89]. Chronic bronchitis is one of the most common features co-existing with **chronic obstructive pulmonary disease (COPD).** Up to half of smokers develop COPD [90] and non-smoking females are more likely to develop COPD than non-smoking men [91].

According to the GOLD document, COPD is a defined as a preventable and treatable disease characterised by persistent respiratory symptoms and airflow limitation due to airway and alveolar abnormalities often caused by exposure to harmful particles and/or gases (GOLD 2018). The diagnosis is based on medical history together with spirometry where $FEV_1/FVC < 0.7$ after bronchodilatation. Lung function, assessed as FEV_1 in percent of predicted value, defines the stage of COPD, from mild to very severe.

Inflammatory cells like neutrophils, macrophages and cytotoxic CD8+ T-cells in combination with epithelial cells are important for the progression of COPD. They induce a cascade of events, including the release of neutrophilic chemoattractant CXCL8, and matrix metalloproteinases (MMPs), which leads to a breakdown of connective tissue and development of emphysema [92]. Many patients with COPD and emphysema are skinny, may have a barrel chest and are often observed leaning forward sitting to facilitate breathing. The most common symptoms in COPD are productive cough, wheezing, breathlessness (dyspnea) and fatigue in more severe disease. During the progression of COPD, acute exacerbations increase in frequency and are often triggered by respiratory infection from viruses, bacteria or environmental pollutants. To ease the constriction, short-(and long-)acting bronchodilators are frequently used in COPD .

Currently COPD is the fourth leading cause of death in the world [93]. In 2012, COPD accounted for 6% of all deaths in the world and most patients suffer for years before dying at premature age. It is estimated to be the third cause of death by 2020 as the population is getting older but also continued or increased exposure to COPD risk factors; tobacco smoking, biomass fuel exposure and air pollution. Occupational exposures to organic and inorganic dust are under-estimated risk factors for developing COPD. In the US, more than 30% of non-smoking COPD cases are linked to occupational exposure and is likely much higher in countries with no or limited regulations for protecting workers' health [93]. Age and low education also constitute risk factors for developing COPD [91]. An additional risk factor is asthma (12-fold increased risk of developing COPD after adjusting for smoking).

Asthma is a common and heterogeneous airway disorder affecting about 300 million people worldwide [94]. The prevalence of asthma differs between countries between 1 and 18%. It is characterized by chronic inflammation of the airways including wheezing, shortness of breath, and cough together with variable airflow limitation. Unlike COPD, the airway obstruction is reversible spontaneously or in response to medication. Allergic asthma is the most recognizable phenotype, often starting in childhood and associated with family history of atopic dermatitis and/or allergy [94]. This type of asthma involves T_h2-cells and eosinophilic recruitment where the latter is dramatically reduced upon treatment with glucocorticosteroids [95]. Non-allergic asthma can be triggered by exercise, infection, aspirin, or cold air. These patients may have an increase in eosinophils, but the increase is less pronounced than in allergic asthma, and they are less responsive to inhaled steroids [94].

Occupational exposure to swine dust induces impaired immune host response among the farmers as they develop **immunological tolerance to organic dust.** This response is similar to LPS or endotoxin tolerance, described first in animal studies and later in patients with e.g. sepsis or trauma, upon repeated exposure to endotoxins [96].

Previously healthy, and never-exposed subjects, who are exposed to swine farm environment, often develop ODTS (organic dust toxic syndrome). It is characterized by flu-like symptoms like chills, fever and malaise that appears a few hours to half a day post exposure and disappears within one to a few days [97]. Never-exposed healthy subjects exposed to swine barns

environment increased both CXCL8 in BALF and nasal lavage fluid, the latter positively correlated to nasal lavage neutrophils [98].

Naïve, healthy volunteers – divided into low and high responders depending on FEV_1 outcome after high endotoxin/dust exposure – had a higher increase in blood lymphocytes, serum IL-6, total nasal lavage cells and nasal lavage CXCL8 in the high responsive group than the less responsive group [99]. When comparing never-exposed healthy volunteers to healthy swine farmers after 3 hours of swine farm exposure, the never-exposed volunteers had a much greater increase in bronchial responsiveness to methacholine and serum IL-6 levels than the swine farmers. Lung function was less affected in swine farmers, as FEV_1 dropped significantly more for volunteers than for farmers post-exposure. Additionally, the volunteers had an increase in the number of nasal lavage cells post-exposure while the farmers had no change. This suggests that the farmers had developed tolerance to the swine farm environment [100, 101].

The blood and sputum differences in expression of PRRs and adhesion proteins among healthy swine farmers and never-exposed, healthy volunteers after swine barn exposure was explored by Sahlander *et al* in 2012 [81]. Farmers showed lower levels of sTLR2 and sCD14 in sputum and reduced expression of CD14 on sputum neutrophils than controls. Systemically, blood monocytes were higher and expression of CD62L and CD162 on blood neutrophils were lower in farmers than in controls. The lack of increase in serum sST2 in farmers compared to swine farmers after LPS and swine dust exposure [81] could be linked to the reduced cytokine response [100] and suggest the development of tolerance development against the effects of organic dust exposure.

When exposing 16 former swine farmers for 3 hours to a swine farm environment, they reacted similarly to active farmers; reduced serum TNF-α, monocyte expression of CD14 and HLA-DR on alveolar macrophages [102] suggesting that endotoxin tolerance persists over time.

In farmers, it was reported that high endotoxin concentrations did not render expected effects like ODTS. The authors speculated that it could be due to non-specificity of the LAL (Limulus amebocyte lysate) assay or that not only endotoxin could cause the effect linked to ODTS [103]. At present, the influence on β-1,3-glucan on LAL assay is known (see Methods). Additionally several studies suggest that peptidoglycan content of the organic dust could be an important contributor to adverse effects in humans [23, 101, 104-106]

Models to study respiratory innate immunity

Generally, in toxicology and in immunology, animals are used for *in vivo* studies to learn more about events that may occur in humans. The use of animals in research has a long history in medicine and toxicology, which led to the discovery of insulin and vaccine and helped screening for carcinogenic drugs and other chemicals. There is increased pressure to replace, reduce and refine animal testing (3R) by regulatory authorities, non-governmental organizations and the public. However, in some instances research on animals might be

required in order to understand systemic effects when animal-free replacements are not available.

Toxicity and effects on innate immunity of airborne substances and mixtures can be characterized using different models [107], from simple cell lines in submerged conditions, or complex organ-like *in vitro* models to animal studies using (knock-out) rodents, as well as controlled human clinical studies, all aiming to understand the hazard(s) to humans. One of the main challenges in respiratory toxicology is mimicking the complex relationship of epithelial cells of the airways combined with the difficulties to expose the cells or organ to a physical form resembling to what could be found in ambient air.

In some cases, findings are first observed in human *in vivo* studies and later "confirmed" by animal *in vivo* studies. For instance, the tolerance to organic dust after repeated exposure was first found in humans [100] and later in rats [108] and mice [109]. Identifying animal models that reflect the human response are useful since they allow for an in-depth evaluation of the disease state, including histopathology, that is not possible with the limited samples available in humans.

Both Paper I and II use human ***in vivo*** studies with a **crossover** design (Figure 2). Typically, a crossover study includes a limited number of subjects, as the power is higher than for instance a case-control or cohort studies as the subjects acts as their own control which reduces the influence of confounders. These advantages should be weighed against the disadvantages including the risk of dropout possibly due to long study duration and the difficulties assessing possible carryover effect.

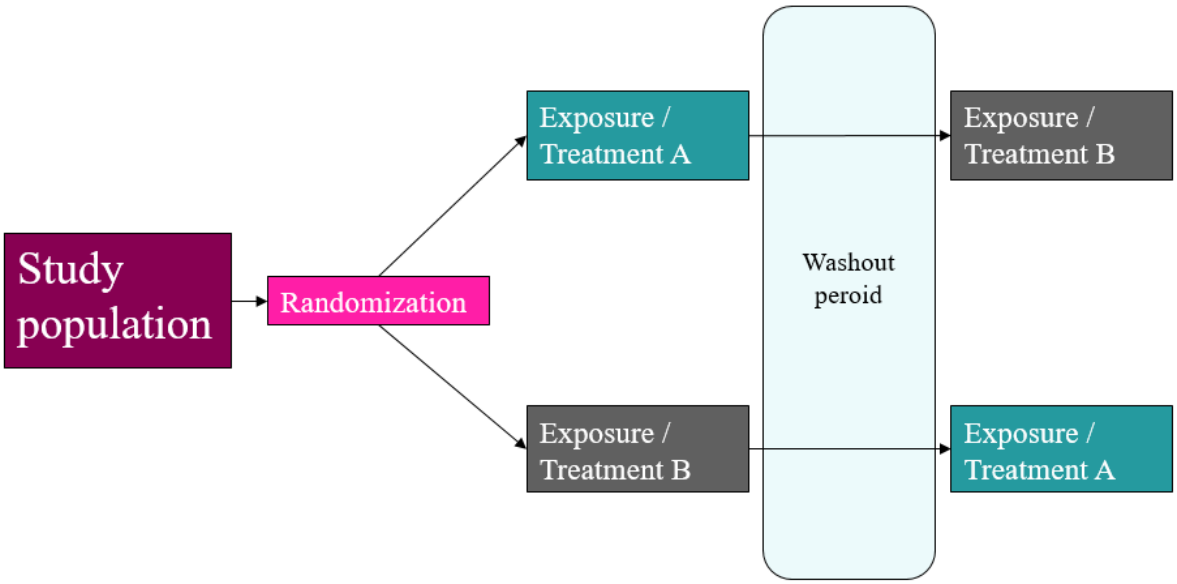


Figure 2. Generic design of a cross-over study.

Different organs and cell types have their own challenges. To study the respiratory tract *in vitro* is challenging due to its complex anatomy including the numerous cell types involved and exposure of xenobiotics via air-liquid interphase. In academia the traditional *in vitro* system for assessing respiratory hazard potential of inhaled (ultrafine) substances is using one cell type and even possibly a cell line which is exposed via the cell culture media into which the (water soluble) substance is diluted. This methodology suffers from many flaws since it is far from mimicking real-life exposure and cell morphology and interactions [110].

Commercial exposure systems are available (CULTEX and Vitrocell®) [111] and several research groups have developed in-house methods including ALICE, EAVES, NACIVT [112]. The methods are often based on electrostatic precipitation of aerosols over the cells. Important disadvantages with these exposure systems are alterations of physical characteristics of the particles by particle aggregation and droplet formation [111].

Recognizing the current limitation, the focus of Paper III was on developing an improved model that employed co-culturing human cells forming a three-dimensional airway epithelia which could be combined with realistic exposure of aerosols in air. This new *in vitro* model could possibly serve as a replacement for future animal studies focusing on local innate immune effects, both acute and long-term.

GLUCOCORTICOSTEROIDS

Glucocorticosteroids are endogenous steroid hormones and synthetic anti-inflammatory drugs used for treatment of chronic inflammation and immune diseases like asthma, rheumatoid arthritis and inflammatory bowel disease. Synthetic steroids therapy was initiated 70 years ago and was awarded a Nobel prize in 1950 for the discovery of treating rheumatoid arthritis.

The drug enters the cell and binds to the glucocorticoid receptor (GR), present in the cytosol of almost all organs and tissues, and is translocated to the nucleus, where it upregulates anti-inflammatory proteins and represses pro-inflammatory proteins in the cytosol [113]. Endogenous steroids, like cortisol, also bind to GR but also to the mineralocorticoid receptor (MR). To avoid adverse effects of the important endogenous systems, drug development has focus on improving the specificity of exogenous steroids to bind to GR. Budesonide and fluticasone are examples of commonly prescribed inhaled corticosteroids (ICS) for treating chronic inflammation in the respiratory tract, like asthma and COPD. Some COPD patients, and asthma patients, respond poorly to ICS. Several mechanisms could be the reason behind ICS resistance. In an attempt to address the issue of resistance researchers and clinicians have tried to identify alternatives but have been hampered by toxicity and unwanted side effects and therefore have identified only a few alternative drugs [113, 114]. In addition, those alternatives they have identified also have limited effectiveness in some patient groups [115]. Phosphorylation of GR via IL-2, IL-4, IL-13, cytokines that are over-expressed in corticosteroid resistant asthmatics, can decrease its activity [116]. Yet the mechanisms of glucocorticosteroids are complex and even possible pro-inflammatory effects have been suggested [117-119].

AIMS OF THE STUDIES

Overall, the aims of the studies were to investigate the host innate immune responses in humans – *in vitro*, *in vivo* and *ex vivo* – after exposure to nano-sized palladium and particulate matter of organic dust present in swine farms.

Paper I

The aim was to compare respiratory effects in healthy subjects after acute exposure to organic dust in swine buildings before and after installing particle separators, which aimed to reduce particulate matter exposure.

Paper II

The aim was to investigate the host innate immune response *in vivo* in chronically organic dust exposed swine farmers after short-term glucocorticosteriods therapy.

The aim was to elucidate the cellular immune response of AM from chronically organic dust exposed swine farmers to *ex vivo* co-stimulation of glucortocosteroids and/or TLR ligands.

Paper III

To develop an organotypic *in vitro* exposure system; combining human bronchial mucosa models with Xpose*ALI*® for exposure of nano-sized palladium.

MATERIALS AND METHODS

MATERIALS

Study designs

In this thesis, both human *in vivo* and *in vitro* models have been used.

Human ***in vivo*** crossover studies (described above in Figure 2):

- Paper I analyse the biological effects of dust reduction intervention in swine stables among healthy volunteers (Figure 3).
- Paper II focus on health effects in swine farmers after budesonide therapy and *ex vivo* stimulations of TLR ligands LPS, petidoglycan or TNF-α with or without budesoinde of swine farmers' alveolar macrophages.

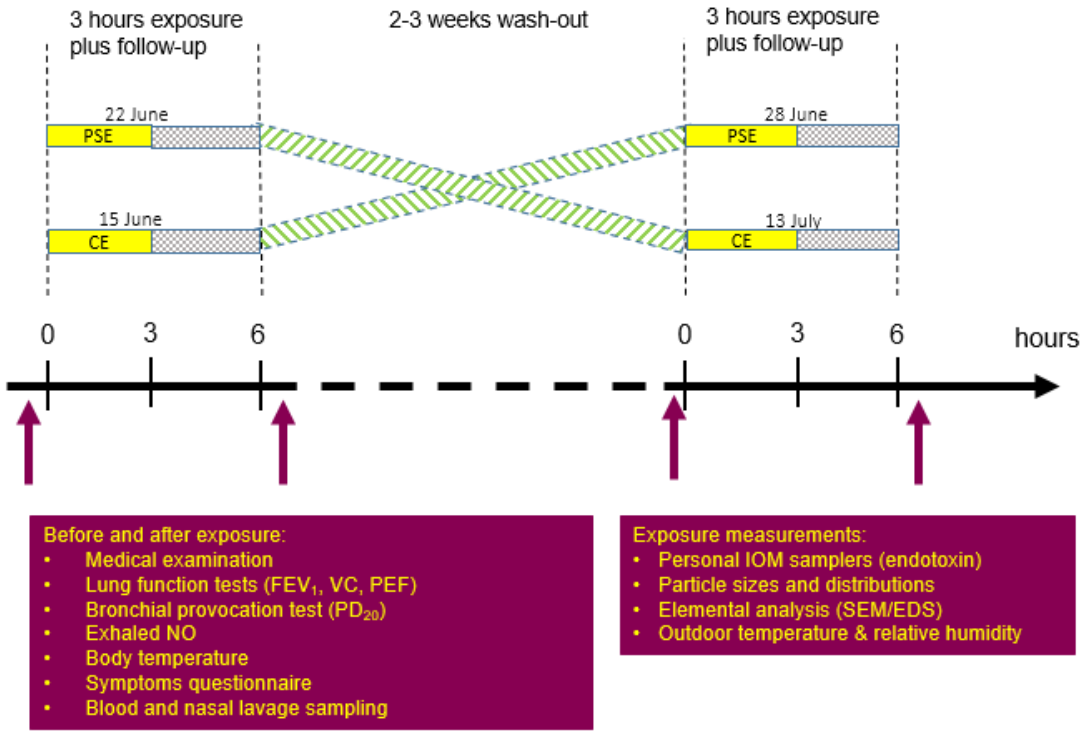


Figure 3. Study design of paper I including perfomred biological and exposure measurements.
CE: conventional swine building environment, PSE: particle separated swine building environment

In vitro 3-dimensional bronchial mucosa model:

- Paper III explains the development of a new *in vitro* model that could possibly serve as a replacement for future animal studies focusing on local innate immunity. It focus on co-culturing human primary cells and cell lines forming a three dimensional airway mucosa combined with a realistic exposure of aerosols present in air.

Human study populations

All subjects gave their informed consent and the studies were approved by the Ethics Committee of Karolinska Institutet.

- Eleven healthy, non-smoking, non-allergic subjects who never had been exposed in swine farms were included in Paper I.
- Fifteeen, healthy, non-smoking, non-allergic swine farmers (>6 months recent occupational exposure) were included in Paper II.
- Alveolar macrophages of a subset of seven swine farmers were used for the *ex vivo* part of Paper II.
- Primary bronchial epithelial cells (passage 3) from healthy tissue taken during lung lobectomy of 3 donors together with a fibroblast cell line (passage 26) were used for study III.

Exposure

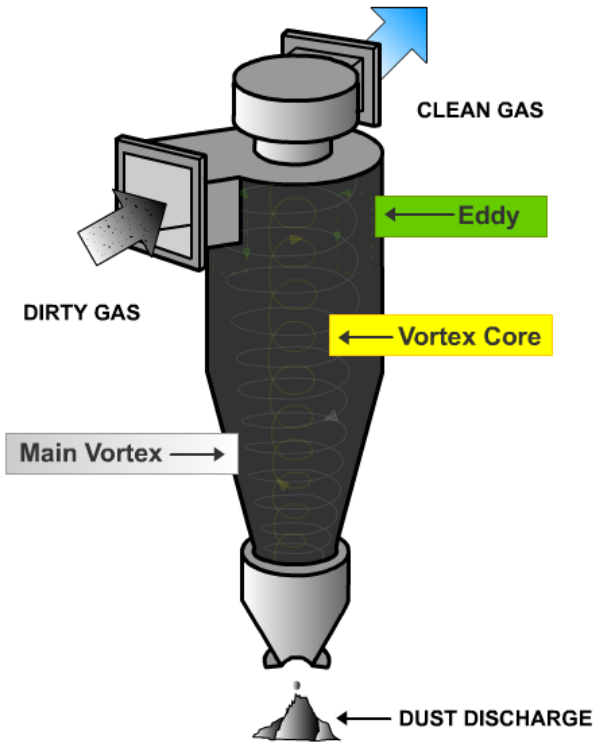
Paper I

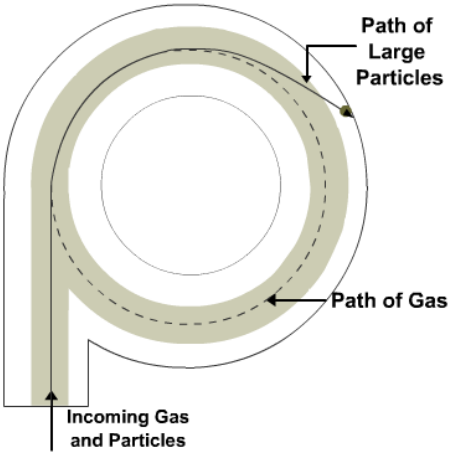
The healthy subjects were exposed twice to organic dust for 3 hours, by being present during the weighing of swine in two swine barns. These barns were identical except for one difference, the presence of installed cyclones with the aim to reduce the ambient dust concentrations in the swine barns air.

The two barns were identical; area of 550 m^2, ventilation air exchange rate of maximum 30 total air volumes/hour, temperature of 20-22 °C, except on hot summer days when stable temperature was about 1-2 degrees warmer than outside temperature, housed 350 swine (weighing 90 kg) held on concreate floor with wood shavings given wet feed.

Pre-installation of the cyclones, both stables were washed clean. Swine were housed 10 weeks before first exposure of the the study subjects who were randomised into two groups and exposed to the two stables for 3 hours between 8 and 11 in the morning, in a cross-over design with 2-3 weeks of washout in between. The stables were called conventional environment (CE) and particle-separated environment (PSE). The subjects were exposed during weighing of the swine, which generally render high dust exposure due to the intense activity of the swine and the farmer.

Four particle separators/cyclones were installed, one in each corner of the the stable (Figure 4), aiming to reduce organic swine dust concentrations of the indoor air. The total air exchanges of the cyclones were 3 times per hour.





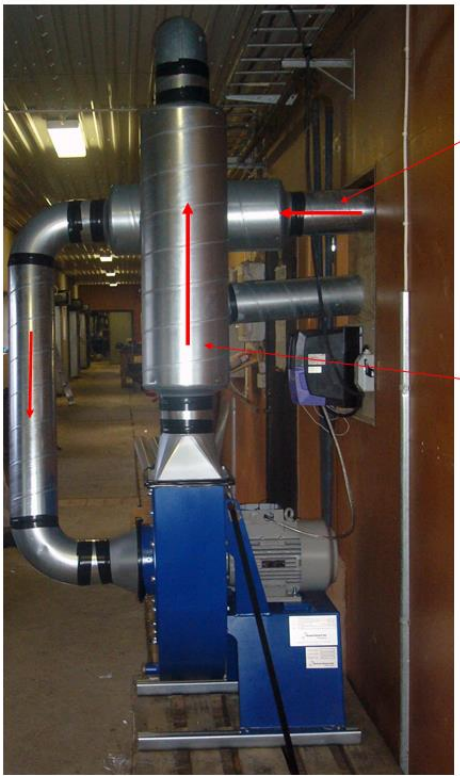


Figure 4. Cyclone design. Generic sketch (above) and the installed Centriclean cyclone and fan below.

Paper II

The swine farmers, chronically occupationally exposed to organic dust in swine barns, inhaled budesonide 400 μg or placebo twice daily for two weeks the with a 4 week washout period in between the two treatments. Throughout the budesonide and placebo treatment periods the farmers continued working as usual in the swine stables. To assure chronic swine dust exposure the farmers had worked at least 6 months in swine environments prior to entering the study.

Alveolar macrophages collected from a subset of 7 farmers were grown in 12-well plates and stimulated for 6 hours *ex vivo* with budesonide (10^{-8} M) and/or co-stimulated with TLR ligands LPS (1 μg/ml), peptidoglycan (1 μg/ml) or TNF-α (10 ng/ml).

Paper III

Two types of co-cultured 3D- cell models were used for the experiments; models from 3 different donors both normal and chronic bronchitis-like models (treated with IL-13 to induce increased number of mucin-producing cells). These models were exposed to palladium nanoparticles sized 6-10 nm using the Xpose*ALI*® module of the PreciseInhale™ exposure system for assuring an even and precise dosing during 3 minutes. The models were assessed 2, 4, 8 or 24h post-exposure.

The PreciseInhale™ exposure platform combined with the Xpose*ALI*® module allows for exposure of cells to respirable sized aerosols (Figure 5). The small sample of palladium was put onto the loading chamber and assembled with the nozzle and the aerosol chamber. A high-pressure air jet of 100-140 bars was shot through the nozzle, which aerosolize the palladium powder into the 300 ml aerosol/holding chamber. The aerosol is further drawn at a speed of 90 ml/min, to pass by the Casella light dispersion instrument before reaching the Xpose*ALI*® module. Here the tube diverts into three consecutive branch flows of 10 ml/min that are connected with the three inserts of co-culture cell models. To calculate the dose of each insert, the signal of the Casella instrument was correlated to the weight of palladium on the end filter. In this paper, the substance correlation factor was 0.8648. To achieve target dose, the duration of the exposure was altered. The exposure times for the different concentrations were; low dose was exposed for 20 seconds, medium dose for 45 seconds and high dose for 3 minutes. This corresponds to 250, 400 and 650 ng palladium/cm^2 insert/cell surface. To investigate the dose, exposed models were dissolved in aqua regia, neutralized to pH 3 and analysed by IPC-MS (inductively coupled plasma-mass spectrophotometer). To control for the manual handling of the inserts outside incubator environment and for the exposure itself, sham cultures were exposed to clean exposure system and only pressurised air. After the exposure, the models were incubated for 8 and 24 hours in 5% CO_2 at 37 °C before apical medium was collected by lavage for 15 minutes and the basal medium from the basal chamber was collected and stored at -80° C until further analysis.

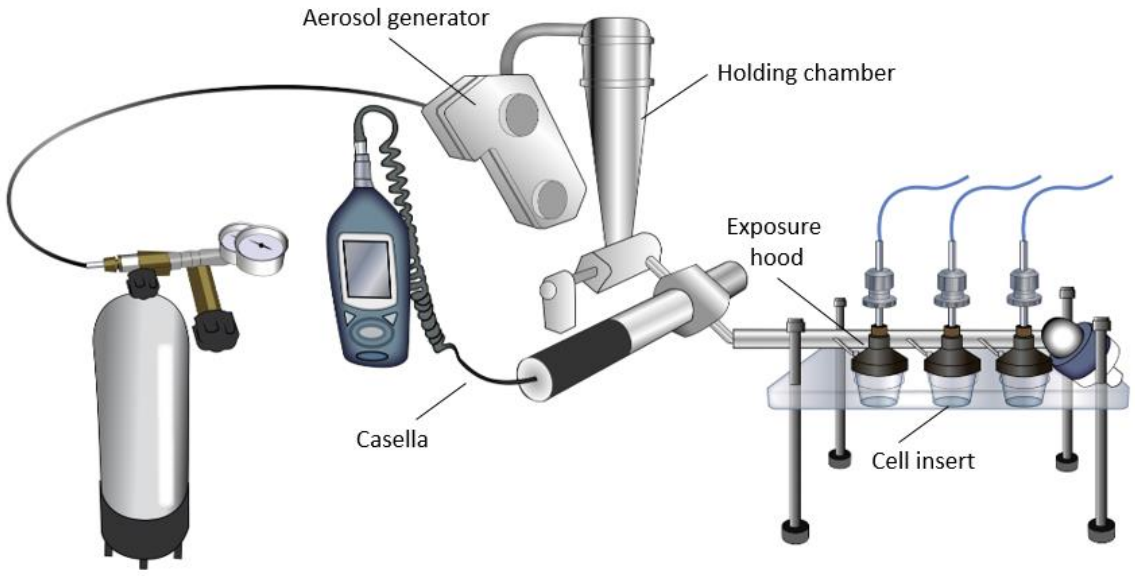


Figure 5. Exposure system: PreciseInhale™ platform including aerosol generator, holding chamber and Casella combined with Xpose*ALI* ® module with exposure hoods and cell inserts.

Sample collection

Overall, samples were collected from included study subjects approximately at the same time of the day, often early in the morning. Not only for practical reasons but also to avoid the influence of circadian rhythm to effect the results. Especially for asthma patients with worsening symptoms during the night often around 4 a.m., many parameters like lung function and exhaled nitric oxide are effected by circadian rhythm [120-122]. Also the innate responses including the cytokine secretion naturally fluctuates during the 24 hours [123].

Spirometry is an important measurement for measurement of ventilation. Trained nurses and general practitioners can perform the analysis, which helps diagnosing asthma, COPD and other lung disorders. The method is simple and quick as well as non-invasive. Yet the analysis require the patient to cooperate and to be able to take instructions from the well trained medical staff. Good reproducibility between measurements is a quality requirement. As lung function is dependent on age, length and weight, the results are also compared to the general population and a percentage of predicted value is calculated.

Spirometry was performed in Paper I and II. In Paper I, we investigated the lung function on three occasions; before and 7 hours after the exposures to swine barn environment with and without particle separators in the stable. In Paper II, we investigated lung function before and after the two two-week treatments with budesonide and placebo. A wedge spirometer was used to measure VC and FEV_1. The same trained nurse was assisting the subjects on all occasions.

Bronchial responsiveness is a term describing the tendency of airways to constrict to direct stimuli such as allergens and non-specific stimuli like cold air, exercise or methacholine. Methacholine binds to the muscarinic M3-receptor in the airway smooth muscle cells, which causes the airways to constrict. The methacholine challenge test is often used to diagnose asthma.

Exhaled nitric oxide (F_ENO) was measured using a non-invasive method that has been used for assessing airway inflammation for almost 30 years [124]. Nitric oxide is produced by most cells both epithelial and endothelial and inflammatory cells in the bronchi and alveoli of the respiratory system. During inflammation nitric oxide synthase is induced and these enzymes can generate nitric oxide while converting L-arginine. Especially increased levels of nitric oxide synthase 2 is found in the airways in asthma and is reduced by steroid treatment. In COPD patients the formation of nitric oxide seems to be linked to a different synthase. Nevertheless, exhaled nitric oxide is a useful tool in understanding eosinophilic inflammation of the airways in e.g. asthma [125]. Important confounders when measuring F_ENO are ingesting nitrate-rich vegetables like spinach [126] and smoking [124]. In Paper I, exhaled nitric oxide was assessed according to the ATS recommendations [121] using a single-breath exhalation with a flow rate of 50 mL/s.

Symptoms questionnaire are an easy, non-invasive measurement, but yet efficient, especially in understanding intra-subject changes over time or treatments. In Paper I and II, the participants were asked to estimate and document body symptoms by marking on a 100 mm line (scale) where 0 was no symptoms and 100 unbearable symptoms. Five general symptoms (chills, headache, fatigue, muscle pain and nausea) and seven airway-specific symptoms (sneezing, stuffy nose, runny nose, coughs, tight chest, shortness of breath and wheezing) were recorded by the participants before and after the exposures. Additionally, for Paper I, the participants were asked to estimate when, over the course of the day, the symptoms were most pronounced.

Blood sampling was performed on the day of medical examination and 6 hours after beginning of each exposure (Paper I) or after 2 weeks of each treatment (Paper II).

Venous blood was collected in different tubes depending on analysis of interest: serum (protein release using ELISA, Paper I and II), heparin (intracellular staining using flow cytometry, Paper II) and EDTA (cell surface markers using flow cytometry, Paper I and II) tubes.

Nasal lavage is simple and rapid to perform and often used for research of the upper respiratory tract. In Paper I, it was performed on the day of medical examination and 6 hours post beginning of each exposure. Sterile 0.9% sodium chloride (5 ml) was instilled into one nostril and 10 s later expelled and collected. The procedure was repeated in the other nostril and the samples were pooled. After centrifugation of the lavaged cells, they were counted and the supernatant was frozen for future analysis of soluble proteins.

Induced sputum is a semi-invasive method to diagnose lower respiratory diseases. It requires the patient and the well-trained nurse or technician to cooperate well. Avoidance of excessive bronchoconstriction is required but emergency bronchodilators and other drugs should always be close [127].

In Paper II, an experienced research nurse executed induced sputum. After salbutamol inhalation, sputum was induced by inhaling increased concentrations of saline whereupon subject was asked to cough deeply and finally make an attempt to expectorate sputum. A sample larger than 1 gram and macroscopically free from saliva was considered sufficient. After filtering and centrifugation, the total cell number and cell viability was determined by Trypan blue and the supernatant was stored at -70°C until further ELISA analysis.

Bronchoalveolar lavage (BAL) is an invasive method that requires sedation and due to this, the method is often not the first measure employed to diagnose a patient [128]. Although not commonly performed, the collection of bronchoalveolar lavage fluid (BALF) is very valuable for research of the mechanisms of respiratory diseases [129]. Additionally, other tissues than BALF could be collected during the bronchoscopy, for instance bronchial brushing of the epithelial wall. In our study II, bronchoscopy and BAL were performed according to established procedures at Karolinska University Hospital [129]. After morphine sedation, a flexible fiber optic bronchoscope was inserted via nose or mouth into the lower airways under local anaesthesia (Xylocain®). Five aliquots of 50 ml of sterile saline solution were instilled into the middle lobe of the right lung, recollected by aspiration and put on ice until further analysis.

Inflammatory mediators from 3D models

Apical medium was collected by lavaging the epithelial layer with 180 µl of medium for 15 minutes. The basal medium was collected from the bottom of the well including the cumulative secretion. Both apical and basal medium were collected after 8 and 24 hours post-exposure for later CXCL8, MMP9 and CC16 analysis.

METHODS

Exposure measurement

Particle characterization, exposure and uptake

In Paper I, scanning electron microscopy/energy dispersive spectroscopy SEM/EDS was used for analysing dust, collected on stubs during the first 20 minutes in the swine farms on the days of exposure. The stubs got saturated despite the short collection time so the analysis was semi-quantitative. Elemental analysis of 150 particles on each stub was performed

In Paper II, palladium nanoparticles were synthesized by Bradley's reaction of solvothermal decomposition of Pd(II)-acetylacetonate [130]. For assessing purity of the palladium nanoparticles SEM/EDS was used.

For the crystallinity assessment of the palladium particles X-ray powder diffraction was used. Palladium size was determined by image analysis using transmission electron microscopy (TEM). The palladium dose was determined by ICP-MS and the palladium post-exposure uptake of the models was detected using TEM after 2, 4, 8 and 24 hours incubation.

Bronchial mucosa model establishment

In Paper III, we develop *in vitro* mucosa-like models; one normal "healthy-like" and one IL-13 stimulated "chronic bronchitis-like" model [45]. This procedure requires approximately 30 days as shown in Figure 6.

To create the models, PBEC were seeded (1×10^5 cells/cm^2) on pre-coated 0.4 µm transwell inserts in a 12-well plate. Complete keratinocyte serum-free medium (KSFM) medium (with all supplements) were added (1 ml) to basal and apical side of the insert and culture medium was changed every other day. After one week, the number of cells reached around 3×10^5 cells/cm^2 and the cells were confluent on the insert. The insert was turned upside down, placed in a sterile petri dish and 100 µl of complete Dulbecco's modified eagle medium (DMEM) medium (with all supplements) containing 1×10^4 cells/ml of fibroblasts was added to the downside of the insert membrane. The fibroblast-containing insert was covered and incubated for 30 min at 37 °C while 50 µl complete DMEM was added every 10 minutes to prevent desiccation. Once fibroblast attached to the membrane, the insert was put back into the twelve-well plates in its normal position with 1 ml complete KSFM medium on both sides of the insert. The model was cultured submerged overnight in incubator to allow the two cell types adapt to each other. To initiate the air-liquid-interface (ALI) culturing of the models, all medium was removed and 800 µl ALI medium (complete KSFM medium supplied with calcium chloride, ethanolamine and retinoic acid) was added to the basolateral chamber. The model is viable in 5 % CO_2 at 37 ºC up to 4 weeks by changing the ALI medium in the basolateral chamber every other day. After 3 weeks of ALI culturing, the cell number of our models reached about 1.4-1.8×10^6 cells/cm^2.

To initiate chronic bronchitis-like models, the models were stimulated by addition of 1 ng/ml and 10 ng/ml recombinant human IL-13 to the ALI medium. All the other procedures were identical to as above.

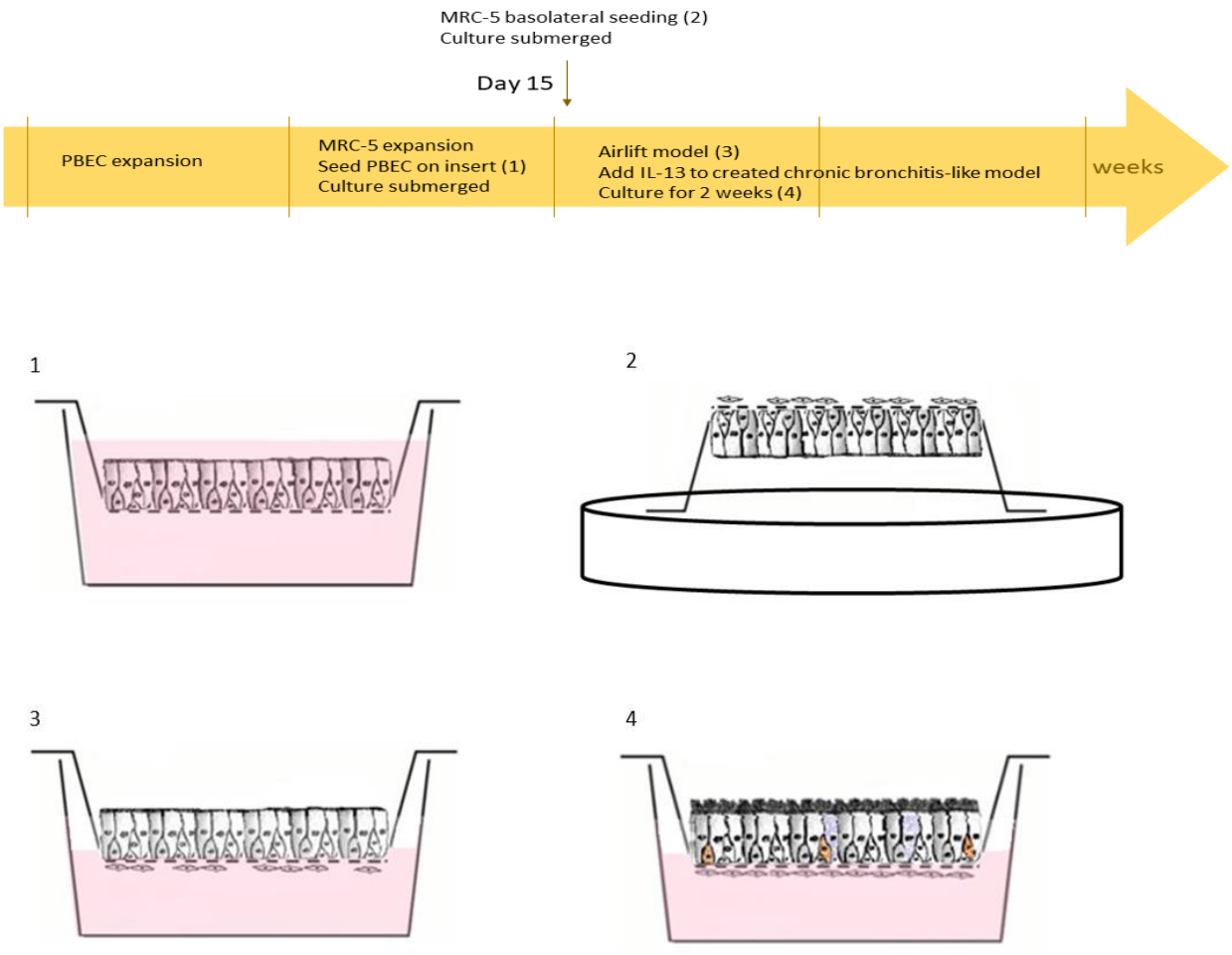


Figure 6. Timeline and main steps in bronchial mucosa model establishment (performed under sterile condition and cultured in 5% CO_2 at 37°C)

1) Apical PBEC seeding in semi-porous 0.4 µm transwell membrane 2) Basolateral seeding of fibroblast MRC-5 3) Removal of medium and medium addition only to basal chamber 4) Cell differentiation during culturing under air-liquid interphase. (Adaptation from [45]).

3D cell model characterization and viability

To assess the morphology of the differentiated co-cultured bronchial mucosa models light-, confocal microscopy, scanning- and transmission electron microscopy and transepithelial electrical resistance (TEER) were used.

For **histological analysis,** the membranes containing the models were cut out of the inserts, fixed, dehydrated, paraffin embedded and sectioned before being stained with hematoxylin and

eosin (H&E) or periodic acid-Schiff (PAS). H&E staining is one of the most commonly used stains used in histopathology for staining nuclei and cytoplasm/extracellular matrix respectively [131]. PAS staining is useful for identifying molecules found in connective tissue and mucus [132].

To assess ciliated cells and mucin producing cells of the models using **confocal microscopy**, the fixed models were stained with mouse anti-acetylated alpha tubulin antibody and rabbit anti-mucin 5AC antibody, respectively followed by secondary antibodies (Alexa Fluor® 488-conjugated goat anti-mouse IgG and Alexa Fluor® 555-conjugated goat anti-rabbit IgG), respectively. The models were mounted on microscope slides with DAPI (4′,6-diamidino-2-phenylindole). Negative control slide was prepared by excluding the primary antibodies. Images were captured and visualized using a LSM700 confocal microscope.

For **scanning electron microscopy**, the model membranes were cut out from the insert and fixed in glutaraldehyde in sodium cacodylate buffer containing sucrose and calcium chloride. The fixed membranes were dehydrated in ethanol and dehydration were completed by critical point drying under CO_2 for best sample morphology preservation. The membranes were mounted and sputter-coated for examination in scanning electron microscope.

For **transmission electron microscopy**, the model membranes were cut from the insert and fixed in glutaraldehyde in sodium cacodylate buffer containing sucrose and calcium chloride. The membranes were post-fixated in osmium tetroxide in sodium cacodylate buffer for 2 hours. After dehydration, the membranes were embedded in LX-112 solution and sectioned using an ultra-microtome. After contrasting the sections with uranyl acetate and lead citrate, they were examined in a Tecnai 12 transmission electron microscope.

To measure the integrity of tight junctions in an *in vitro* system, the non-invasive **transepithelial electrical resistance** (TEER) is a reliable and sensitive method [133]. In study III, EVOM voltage-ohm meter and chopstick electrodes were used. The TEER values were calculated from the mean of 8 inserts per condition, subtracting the TEER value from an insert without cells and multiplied by membrane growth of 0.9 cm^2.

The PBEC viability was assessed by **trypan blue staining**. Trypsinised cells in suspension (10 µl) was mixed with 10 µl 0.4% trypan blue and 200 cells were counted to determine viability. To assess the models, 200 µl 0.2% trypan blue was added on top of the models. 200 cells in four sections of the model was counted to determine the viability. PE-annexinV/7-AAD-staining kit stained 2,000 cells from the trypsinised models and the **apoptotic rate** was detected using flow cytometry.

Analysis of proteins

In all the papers of this thesis sandwich enzyme-linked immunosorbent assay (**ELISA**) was used to detect the soluble proteins of the samples. The method requires two antibodies, first the microplate was coated with capture antibody, second the standard and samples were added, and finally the enzyme-linked detection antibody. Once the stopping substrate was added, the

colour in the microplate well is proportionate with the antigen concentration of the sample. The methodology have a high specificity and is sensitive (low detection limits) and is suitable for complex matrices like serum, sputum, BALF and cell culture supernatant. Duo-set ELISA kits from R&D Systems and in-house methods (IL-6 and CXCL8) were used to detect the following proteins concentrations: CXCL8 (Paper I, II & III), sST2 (Paper I, II), IL-6 (Paper I), sTLR2, sCD14, IL-13 (Paper II) and MMP9 and CC16 (Paper III).

Flow cytometry uses multiple laser beams of different wavelength to measure physical and chemical properties of individual cells from blood, sputum, BALF etc. The cells of interest are stained with fluorescent labels and diluted in sheath fluid before injection into the flow cell and pass the lasers in a narrow cell-wide channel. The laser excites the labels of the cells causing them to emit light of a certain wavelength. Multiple filters detect cell size (forward scatter, FSC), granularity (side scatter, SSC) and intensity of the fluorochrome-conjugated antibodies. Both the number of the positively labelled cells and the mean (or median) fluorescence intensity can be detected. Throughout the studies the group changed from using an old, robust and "simple" BD FACSCalibur™ (Paper I) to a high-tech multi-colour LSRFortessa™ (Paper II and III), now allowing usage of complex panels and advanced fluorochromes.

Intracellular expression of cytokines and chemokines in the cell cytoplasm or nucleus can also be detected using flow cytometry as performed in Paper II. Here, the T-cell polarization was of interest hence expression of anti-inflammatory IL-13 and IL-4 and pro-inflammatory IL-2 and IFN-γ were analysed. To increase the intracellular expression of cytokines the blood was first stimulated by PMA and ionomycin. To hinder the cytokines to be secreted from the blood cells they were treated with brefeldin A that inhibited the protein transport by redistributing proteins from the Golgi complex to the endoplasmic reticulum [134]. After fixation and permeabilisation of the plasma membrane the cells are stained with cytokine antibodies and then analysed by flow cytometry using BD LSRFortessa™.

Exposure measurements of endotoxin and dust were performed (Paper I) using portable pumps with IOM filter cassettes equipped with Teflon filters. The samplers were placed in the breathing zones on three participants at an airflow rate of 1.9–2.0 L/min. The filters were extracted in sterile water and the endotoxin concentration was analysed.

Analysis of endotoxins is based on kinetic-chromogenic technique and performed using the Limulus Amebocyte Lysate (LAL) assay from Charles River Laboratories, USA. It uses enzyme in isolated amebocyte cells from Atlantic horseshoe crabs that upon endotoxin induction can cleave the colorimetric substrate [135]. The methodology is analytically straightforward but very sensitive. The methodology is developed for assuring safe pharma so the detection limit is extremely low (0.1 EU/ml). Achieving trust-worthy, repeatable results is challenging especially in high-containing samples like swine dust where endotoxin is likely bound to the complex dust particles and requires extensive dilution for measurable results [21, 136, 137]. Interference of fungal β-1,3-glucan, present in swine dust, is another challenge [135] that was overcome in paper I by using endotoxin specific buffer containing carboxymethylated

curdlan that block the reaction of β-1,3-glucan with LAL (BG120, Charles River Laboratories, USA).

For valid results in Paper I, beyond using non-contaminated lab ware including depyrogenated borosilicate test tubes, special 96-well polystyrene plates and endotoxin-free water samples, the samples required vigorous vortexing and stable hot plate to assure sample temperatures of 37 °C.

Analysis of mRNA

Polymeras chain reaction (**PCR)** amplifies DNA or RNA into measurable amounts.

In our studies we extracted total RNA from the samples (alveolar macrophages stimulated *ex vivo* in Paper II and 3D model PBEC stimulated *in vitro* in Paper III) and assessed its quality by measuring spectrophotometric absorbance ratio at 260/280 nm. First-stranded cDNA was synthesized by revere transcription of total RNA, from which reverse transcription PCR was performed including the primers of interest. Results were adjusted for an internal control gene and expressed as ΔΔCt (relative values) and presented as fold differences compared to the experimental control where ΔCt=Ct (gene of interest) – Ct (internal control gene).

Paper II used RT-PCR to understand the impact of *in vivo* budesonide treatment and *ex vivo* stimulation of TLR ligands and/or budesonide in swine farmers' alveolar macrophages mRNA expression on common pattern recognition receptors and inflammatory mediators. Here we analysed the transcript of genes involved in pro-inflammation (*CXCL8*), innate immunity (*TLR2, TLR4, CD14, LL37*), mucus (hyper)secretion *(IL13, SLPI*), oxidative stress (glutathione peroxidase (*GPx), SOD3*) and tissue repair (*TIMP*). Ribosomal protein L32 (*RPL32*) was used as internal control gene.

Paper III used RT-PCR to validate the differentiation of cells into a mucosa-like model. Here we analysed forkhead box protein J1 (*FOXJ1*) a transcription factor involved in ciliogenesis, *MUC5AC* expressed by mucus producing cells, club cell protein (*CC10/CC16*) produced by non-ciliated club cells and keratin-5 (*KRT5*) that is expressed primarily in basal cells. Beta-actin was used as internal control gene.

Statistics

Non-normal (or unknown distribution) distributed data was presented as median with 25-75th percentiles. Lung function parameters, symptoms and body temperature were considered normally distributed and presented as mean values and 95% CI.

Statistical within group comparisons were assessed by the Friedman test followed by the Wilcoxon signed rank test when appropriate.

Statistical analyses for all papers were performed using STATISTICA (StatSoft).

In Paper I, to analyse symptoms, pre-exposure data from the same morning were deducted from post-exposure data for the two exposure occasions and the differences compared.

In Paper II, correlations between biomarkers and lung function were assessed using Spearman rank correlation test. Student t-test was used for determining the influence of carryover between the treatments. In this paper also the statistical analysis software STATA® was also used.

RESULTS AND DISCUSSIONS

PAPER I

The aim of this study was to compare respiratory effects in healthy volunteers after acute exposure to organic dust in swine buildings before and after installing particle separators, also called cyclones, aimed to reduce dust exposure (PSE: particle separated swine building environment and CE: conventional swine building environment).

The exposure of the subjects were performed at four different days during June and July. Climate data collected by Swedish Meteorological and Hydrological Institute revealed temperature differences, from 14 to 30 °C during the study period (Table 1). The relative humidity was approximately 45 % except for the coldest day when it was 65 %. These meteorological parameters could influence both the outdoor and indoor air conditions and the ability to form and hold particles in the air [138, 139].

Table 1. Outdoor conditions and number of particles per size fraction on days of exposure [105].

	PSE		CE	
	22 June	28 June	15 June	13 July
Outdoor conditions at 11:00				
Temperature (°C)	20.0	21.4	13.6	29.9
Relative humidity (%)	44	45	65	47
Particle number (TWA)				
0.3 to 0.5 μm	29 531	26 208	72 024	76 129
0.5 to 1 μm	3694	1223	29 539	2609
1 to 5 μm	1009	686	20 005	1972
5 to 10 μm	50	243	101	587
10 to 25 μm	48	294	19	590
>25 μm	47	83	11	181

CE, conventional swine building environment; PSE, particle-separated swine building environment; TWA, Time-weighted average during exposure 8:00–11:00.

Endotoxin was measured by three portable devices at the four days of exposure. The inter-filter variability, as well as the intra-filter variability as measured in the aliquots, was large and ranged from below detection limit to 144 EU/ml. It is clear from the literature and from performing these LAL analysis that it is optimized for very low endotoxin concentrations typically found when assuring endotoxin free pharmaceuticals and not for high concentrations found in agricultural environments [137]. Round-robin analysis have, despite using the same or different protocols, shown difficulties in achieving similar results [136]. Yet the results give an indication of the presence of endotoxins in swine building environment. Whether particle separators were used or not, seemed not to be important for the endotoxin concentrations in air. Temperature appeared to influence the endotoxin concentration; the higher the temperature the lower the endotoxin concentration (Figure 7A). Even if the stables are ventilated, there is no air-conditioning why hot outdoor temperatures around 30°C are also reflected in hot indoor temperatures. Temperature also influences the physical activity of the swine and on hot days, the swine tend to lay still on the cold concrete floor which could likely reduce the endotoxin

levels. Low endotoxin levels during warm days have been reported in Danish dairy farms [20] and during summer vs winter in Dutch [140] and Canadian [141] swine farms.

When comparing the two stables (PSE and CE) in terms of particle number differences, the installation of the cyclones reduced the particle fraction sized 0.3-0.5 µm the most without being impacted by the 15 °C difference in outdoor temperature (Figure 7B). Also the slightly larger fractions 0.5-1 µm and 1-5 µm reduced in numbers in the PSE compared to CE. For these fractions in CE, the temperature seemed to influence the numbers as the cold day had much higher particle numbers than the warm day (Table 1). This could again be influenced by the ventilation of the stables as the warmer the stable the higher the ventilation rate. Increasing the ventilation possibly reduce the smaller airborne particles better than the larger (that tend to settle faster to the ground). Yet the smallest particles (0.3-05 µm) could be too small to be affected by the ventilation and being airborne for the longest time they could reach the particle separators.

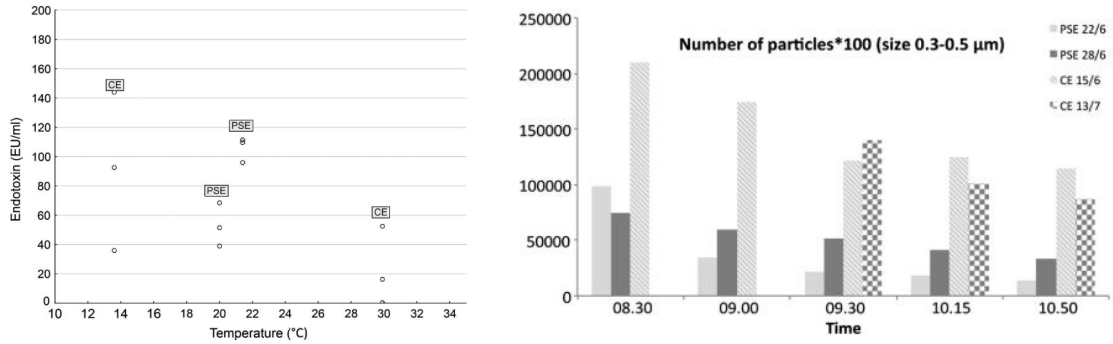


Figure 7. A: Endotoxin concentrations and outdoor temperature on the days of exposure. Three IOM samplers were used on each of the 4 days of exposure, to collect endotoxin samples. B: Particle numbers, sized 0.3–0.5 mm, measured over time in the swine buildings during exposure (dates included). CE: conventional swine building environment, PSE: particle separated swine building environment. [105]

Lung function (FEV_1, VC) and bronchial responsiveness (PD_{20FEV1}, PC_{20FEV1}) were clearly reduced by the acute exposure to the two swine buildings (Table 2), yet with no difference between CE and PSE.

Exhaled nitric oxide increased after both exposures in PSE and CE with no difference between the groups (Table 2). Previously, in similarly exposed healthy subjects nitric oxide levels in exhaled air was doubled, which could be prevented by wearing a half-mask with particle filter. The personal protective equipment did not protect from the increase in bronchial responsiveness [142]. In a more recent study [34], wearing different types of face-masks did not protect from the increase in NO caused by 3 hours exposure in swine barns. This conflicting finding could be explained by the late follow-up (7 hours after initiating exposure) or by lower airborne inhalable dust concentrations. In all, this suggests that different particles are responsible for different mechanisms of action during respiratory inflammation.

Table 2. Lung function data and exhaled nitric oxide before and after the two exposures (CE: Conventional swine house environment, PSE: Particle separated swine house environment). Data are presented as mean and 95% confidence interval. Statistical significance is considered $p<0.05$, n=11.

.Lung function parameters	Pre exposure	Post CE	Post PSE	P-values (pre exposure - CE pre exposure – PSE CE - PSE)
FEV_1 (liter)	4.17 (3.73-4.60)	4.05 (3.58-4.51)	4.03 (3.59-4.47)	0.02 0.009 0.79
FEV_1 (% predicted value)	99 (94-104)	96 (91-101)	96 (90-101)	0.02 0.01 0.86
VC (liter)	4.68 (4.15-5.22)	4.60 (4.04-5.16)	4.53 (3.99-5.06)	0.01 0.01 0.26
VC (% predicted value)	91 (85-97)	90 (83-96)	88 (83-93)	0.01 0.01 0.20
PD_{20} (mg)	3.81 (0.47-7.15)	1.95 (-1.84-5.73)	1.85 (-1.53-5.23)	0.008 0.005 0.86
PC_{20} (mg/ml)	8.16 (1.65-14.7)	4.00 (-3.29-11.30)	3.84 (-2.68-10.35)	0.008 0.005 0.95
NO (ppb)	14.05 (11.53-16.56)	19.68 (16.67-22.70)	19.55 (17.13-21.96)	0.01 0.005 0.86

Analysing blood leucocytes and nasal lavage fluid cells, all but blood eosinophils increased in number after the two exposures ($p\leq0.013$) but with no difference between CE and PSE (NAL cells; Figure 8). Also the ratio of CD4/CD8+ blood lymphocytes increased after CE exposure ($p=0.05$) but without any difference between the two exposures.

After the CE exposure, TLR2 and TLR4 expression on blood monocytes increased ($p=0.016$ and $p=0.017$ respectively) whereas CD14 was not altered. On blood neutrophils the surface expression of CD14 was significantly reduced after CE ($p=0.008$) and PSE ($p=0.003$) exposures. No differences between CE and PSE were observed for the surface expression of TLR2, TLR4 or CD14 on blood monocytes or neutrophils.

Similarly sCD14 in serum was reduced after CE exposure compared to pre-exposure ($p=0.05$) without any difference between the CE and PSE. Soluble ST2 in serum was not affected by the exposures and below detection limit in nasal lavage fluid.

The cyclones clearly reduced the smallest of the ambient swine dust particles. This reduction could possibly be linked to the altered host innate response of the nose where both pro-

inflammatory mediators IL-6 and CXCL8 increased after the two exposures ($p \leq 0.03$) but significantly more after CE exposure than PSE exposure ($p=0.02$ and $p=0.04$ respectively) (Figure 8). In serum, IL-6 and CXCL8 were below detection limit.

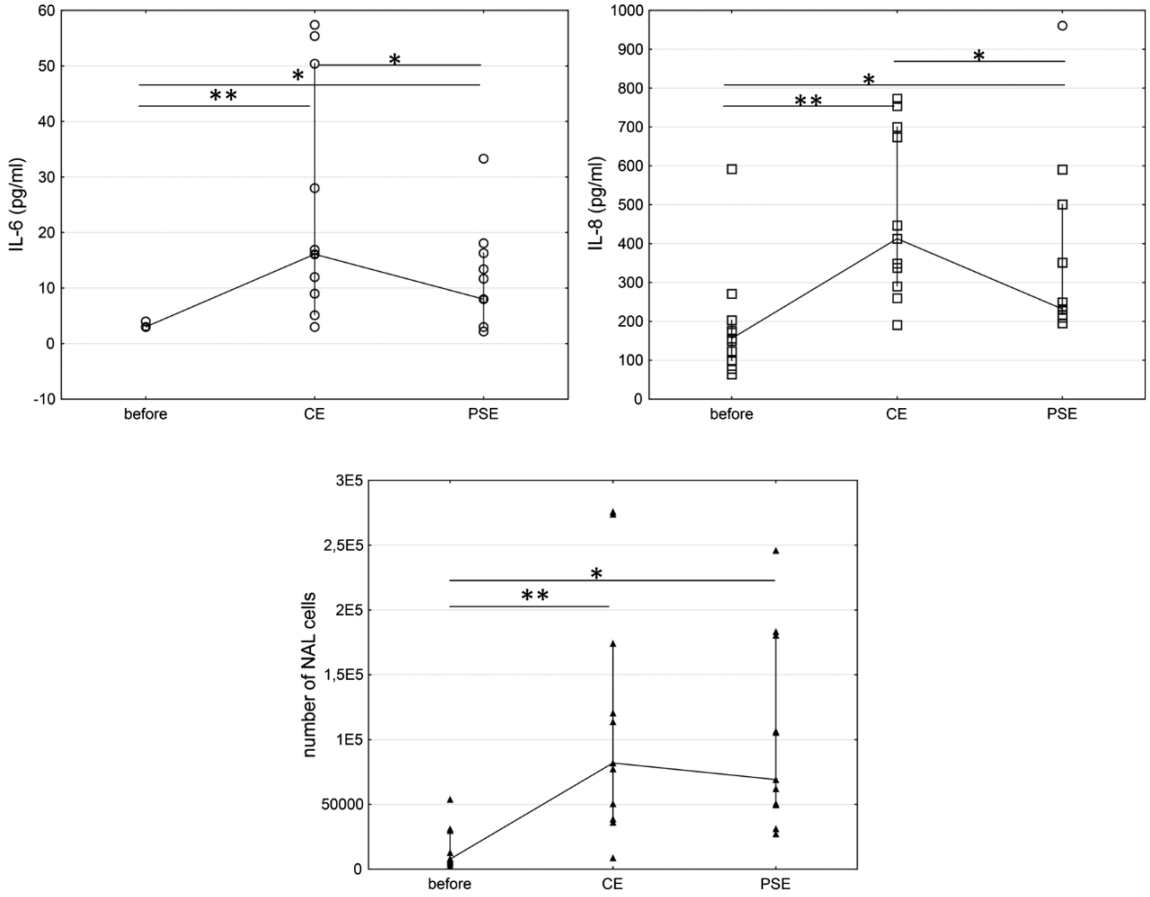


Figure 8. Individual data of IL-6 (open circles) and IL-8 (open squares) in NAL and number of NAL cells (closed triangles). Medians are connected before and after exposure in CE and PSE. *p<0.05, **p<0.01, n=11. IL: interleukin, NAL: nasal lavage, CE: conventional swine building environment, PSE: particle separated swine building environment. [105]

The symptoms questionnaire showed that almost all symptoms increased after exposure to the swine building environments. After CE, the subjects reported more pronounced symptoms directly after three hours exposure and still after another three hours. After PSE, the subjects also reported increased symptoms but the duration of the symptoms were shorter and often back to baseline after six hour from the start of the exposure. Headache was the only symptoms where CE gave significantly higher VAS score than PSE ($p=0.03$). Body temperature measured orally was significantly higher after CE exposure than PSE exposure ($p=0.016$). Compared to the local effects of IL-6 and CXCL8 in the nose described above, headache and body temperature are systemic effects that apparently also were affected by the lower concentration of fine particles (0.3-0.5 µm). Increased body temperature and induced innate immune system is closely linked [143]. Rodents injected with LPS induced TLR4 and release prostaglandin E2 and IL-6 which travels through the blood-brain barrier to initiate fever. Systemic IL-6 was not detectable in serum in this study. In non-naïve but currently non-farmers IL-6 was detected at

its peak in blood one hour after 3 hours of exposure to swine dust in a swine farm occupational setting [102]. The analysis used by Hoffmann *et al* was slightly more sensitive than the method that we used (detection limit of 0.7 pg/ml compared to 3 pg/ml). In our study the blood samples were taken 6 hours after the start of the exposure which could have been too late to find the peak levels combined with natural low levels of plasma IL-6 concentrations around noon because of the circadian variation [144]. Similarly, the IL-6 peak appeared 1-2 hour after the 3 hour exposure when detecting IL-6 in volunteers exposed to organic dust in swine buildings [100, 145]. In our study, the surface expression of TLR4 and TLR2 on monocytes were increased after CE and not PSE, which could be linked to the higher body temperature even if IL-6 could not be detected systemically.

The biological host response was reduced among the volunteers in the particles separated swine farm. One of the variables that we considered was whether the size of the particles had an impact on the response, as it was observed that there was fewer particles in the fine fraction when the cyclones were used. Thus, the number of available particles able to interact with the respiratory system and each cell and PRRs was reduced. Upon particle exposure, the cells in the respiratory tract are stimulated to produce reactive oxidative or nitrogen species which could trigger the release of pro-inflammatory IL-6, CXCL8 and TNF-α. Alternatively, indirectly the particles can induce apoptosis. DAMPs will be released by the dying cells which would bind to TLRs and consequently initiate the release of cytokines and chemokines. IL-6 induces acute phase proteins production including C reactive protein that also could trigger increased body temperature.

Alternatively, the reduced innate immunity response observed in PSE compared to CE could be linked to exposure to chemicals or biological agents that we have not measured in this study. In previous studies, peptidoglycan seem to be linked to adverse health effects post swine dust exposure [13, 23, 34, 104].

The cyclones possibly reduced the fine particles but in order to be even more efficient a few suggestions are made. The total air exchange of the cyclones are much less than the ordinary ventilation especially on hot days, yet the systems work against each other. During the colder seasons when the ventilation systems are less used to save indoor heat and energy, the cyclones are probably much more efficient. During this time of year, endotoxins levels in the air is also known to be higher than during warmer seasons. Additionally, to be able to turn on and off the cyclones when required would save energy and cost for the user and likely increase the effectiveness of the cyclones.

PAPER II

The aim of this paper was to explore the *in vivo* effects of inhaled budesonide (single-blind, crossover design) on host innate immune response in swine farmers who are chronically exposed to organic dust and to further investigate the alveolar macrophages immune response after *ex vivo* co-stimulation with TLR-ligands and/or glucocorticosteroids.

Swine farmers' daily exposure to pro-inflammatory organic swine barn dust with its complex composition of feed, dander, faeces and endotoxin- and peptidoglycan releasing microorganisms induces respiratory symptoms such as cough and increased mucus production. This chronic exposure might contribute to the down regulation of innate immune responsiveness which previously has been shown in farmers. Swine farmers have an attenuated systemic inflammatory response to organic dust exposure in pig barns as compared to healthy non-farmers, including down-regulated TLR2 on blood monocytes [24], significantly decreased sTLR2 in sputum [81] and lack of IL-6 [24, 100] and sST2 increase in serum [81], after exposure to swine dust. The airway responses to pig barn dust exposure are altered in swine farmers compared with healthy non-farmers. Thus bronchial responsiveness and FEV_1 are not reduced to the same extent, exhaled nitric oxide does not increase, IL-6 release in sputum increase less and NAL cell count is not increased after swine barn exposure in the farmers when compared with healthy non-farmers [100, 101]. By treating the farmers with budesonide, the hypothesis was to induce their innate response. Bronchial epithelial cells have shown synergistically increased TLR2 expression upon co-stimulation of budesonide and TLR ligands *in vitro* [146]. Similar results were observed when stimulating alveolar macrophages from smokers with and without COPD with budesonide and TLR ligands [69].

The recruited cohort of swine farmers included more men than women. Also the sub-set of farmers that underwent bronchial alveolar lavage showed heterogeneous characteristic in a similar way as the larger cohort. In the recruited cohort of swine farmers, all were working in confined swine buildings and had median accumulated exposure to swine farming environment of 20 years, spreading from 2 to 39 years, with a daily median exposure of 5 hours (Table 3). Even if no exposure measurement were performed it is clear, based on data in previous studies [15, 100, 105], that the farmers were exposed to organic dust via their occupation and work tasks that are required as swine farmer including cleaning of stables, feeding and weighing of swine. Possibly two of the farmers may have overestimated exposure as they since some years (after many years of conventional farming) produce pigs according to the Swedish rules for organic production (KRAV®). These rules require the swine to be outdoors for 4 months per year, which could possibly reduce the swine farmers' exposure to a confined, indoor space during the summer months. Hoffman *et al*, 2005, showed that even former swine farmers with no current exposure to organic swine dust react similarly to active farmers upon renewed exposure [102]. Therefore, the impact on the biological responses of these two KRAV®-farmers is possibly low and the results are likely not altered due to current production system.

Table 3. Characteristics of the participants. Data presented as median values and ranges except lung function data which are presented as mean (95% confidence interval). FEV_1: Forced expiratory volume during 1 second. VC: Vital Capacity FVC: Forced vital capacity. Exposure is estimated work-related exposure to swine dust and swine house environment.

	All farmers (n=15)	Subset of farmers (n=7)
Age (years)	38 (26-63)	34 (26-53)
Length (cm)	178 (164-190)	175 (164-187)
Weight (kg)	82 (57-113)	80 (57-113)
Gender (f/m)	5/10	3 /4
FEV_1 (liter)	4.01 (3.48-4.54)	3.99 (3.67-4.34)
FEV_1 (% predicted value)	98.4 (90.8-106.0)	99.1 (90.2-108.7)
VC (liter)	4.85 (4.24-5.46)	4.69 (4.22-5.20)
VC (% predicted value)	92.1 (85.5-98.6)	91.1 (81.6-101.4)
FVC (liter)	4.87 (4.27-5.48)	4.75 (4.28-5.26)
FVC (% predicted value)	93.6 (86.8-100.4)	93.3 (83.0-104.4)
Accumulated exposure (years)	20 (2-39)	12 (2-30)
Weekly exposure (h/week)	19 (14-40)	25 (14-40)
Daily exposure (h/day)	5 (2-8)	5 (2-8)
Farm size (no of swine)	1050 (120-3500)	1125 (120-3500)

In vivo

The healthy farmers had normal lung functions which did not change after inhalation of budesonide or placebo. On the contrary, FEV_1 was reduced in healthy non-farmers after exposure to organic swine dust [100]. In addition, the self-reported symptoms of the farmers did not change after the two treatments. In general the farmers reported weak (score 5-10 of 100) cough, nose secretion, stuffed nose and fatigue.

In sputum, sTLR2 and sCD14 are lower in farmers than in healthy controls [81]. In this study where we investigate the effects of budesonide in chronically exposed farmers, sputum cells were not altered during inhalations of budesonide or placebo and none of the soluble proteins measured (sTLR2 and sCD14) differed between the two treatments. Significant increase of sTLR2 was noted comparing before and budesonide treatment ($p=0.002$, $n=15$) (Table 4). Even if the concentrations of sTLR2 increased in sputum post budesonide treatment, swine farmers have still lower sTLR2 concentrations in sputum, than healthy control subjects; sTLR2 sputum concentrations: swine farmers<controls<smokers <COPD patients [81]. In this study, soluble ST2 was below detection limit.

Table 4. Soluble protein release of TLR2, CD14 and ST2 in sputum, serum and bronchoalveolar lavage fluid (BALF). Median and 25-75%. All data presented as pg/ml, except sCD14 in serum which is presented as ng/ml.

		Baseline	After budesonide	After placebo	p-values baseline-bud baseline-plac bud-placebo
Sputum (n=15)					
(pg/ml)	sTLR2	475 (100-665)	611 (100-1047)	445 (100-921)	0.002 0.227 0.135
(pg/ml)	sCD14	6475 (1020-8508)	5757 (1730-8186)	5015 (3121-8665)	0.281 0.235 0.570
Serum (n=14)					
(pg/ml)	sTLR2	1234 (541-3365)	1269 (491-3156)	1289 (555-2970)	0.103 0.186 0.900
(ng/ml)	sCD14	1124 (1046-1257)	1167 (1039-1269)	1124 (1009-1318)	0.510 0.510 0.272
(pg/ml)	sST2	63 (46-81)	63 (46-75)	57 (47-77)	0.148 0.131 0.470
BALF (n=6)					
(pg/ml)	sTLR2	304 (242-487)	347 (243-394)		0.600
(pg/ml)	sCD14	3611 (2763-3925)	3895 (3689-4184)		0.463

Analysing blood (n=14) revealed that all blood cells (lymphocytes (p=0.041), basophils (p=0.048), eosinophils (p=0.004) and monocytes (p=0.041) except for neutrophils (p=0.074) increased in numbers after budesonide treatment compared to baseline but with no difference to placebo treatment. Also CD3+-cells increased (p=0.026) after budesonide compared to before with no difference to placebo, whereas CD8+ or CD4+ were not altered (Table 5).

Table 5. Concentration of leucocytes and the T-cell subset in peripheral blood of the swine farmers before and after budesonide and placebo treatment, expressed as number of cells $\times 10^6$/L blood. (n=14).

Cell type	Before	Budesonide	Placebo	p-value Bud-placebo
Neutrophils	3142 (2404-3771)	3569 (3012-5089)	3487 (3059-4400)	0.433
Monocytes	305 (205-375)	401 (320-502)	357 (262-429)	0.637
Lymphocytes	1613 (1114-1971)	1797 (1431-2202)	1804 (1414-2079)	0.925
Basophils	39 (22-50)	53 (39-64)	50 (35-67)	0.975
Eosinophils	137 (79-150)	185 (141-229)	188 (142-251)	0.551
CD3	1165 (641-1406)	1402 (1129-1746)	1331 (1083-1512)	0.975
CD4	942 (518-1252)	1200 (1074-1397)	1166 (819-1393)	0.875
CD8	351 (258-480)	431 (342-539)	471 (264-511)	0.875
CD4/CD8	2.66 (2.41-3.01)	2.96 (2.41-3.44)	2.79 (2.43-3.07)	0.064

Attenuated systemic inflammatory response in swine farmers have been shown in previous studies including down-regulation of TLR2 on blood monocytes [81]. Nevertheless, budesonide and placebo treatments in our study did not significantly change the expression of TLR2, TLR4 and CD14 expression on blood neutrophils and monocytes. Surface expression of CD14 on lymphocytes was below detection limit. Instead the TLR4 expression on lymphocytes doubled after budesonide treatment compared to both placebo and baseline (p=0.006 and 0.019 respectively, Figure 9).

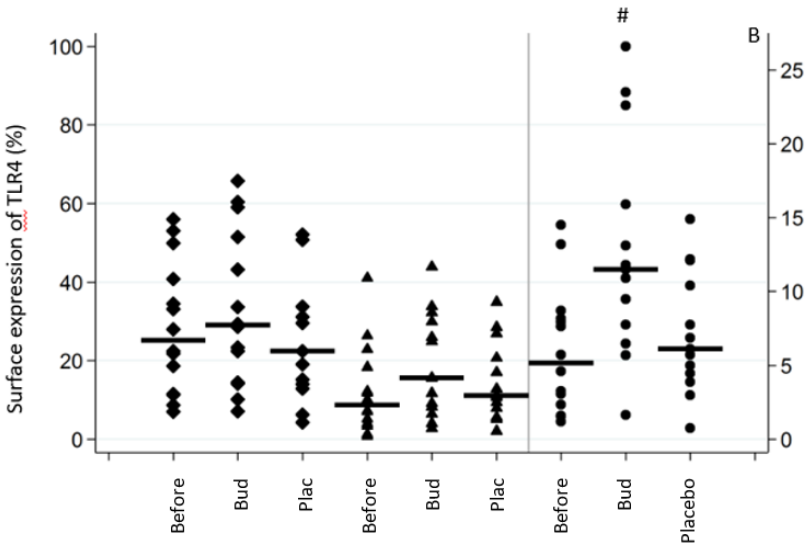


Figure 9. Surface expression of TLR4 (%) on monocytes (diamonds), neutrophils (triangles) and lymphocytes (circles) in peripheral blood of swine farmers at baseline and after inhalation of budesonide and placebo. # $p<0.05$ comparing budesonide treatment with before and placebo (n=14).

When previously comparing farmers and healthy subjects, serum sCD14 and sST2 did not differ [81]. In the present study, treatment with budesonide or placebo did not significantly change the levels in serum of soluble TLR2, sCD14 and sST2 and CRP. The CRP median before treatment was 1.64 mg/L for the farmers, a level considered normal showing no signs of on-going systemic inflammation.

The TLR2 expression could also be influenced by the proportion of different T lymphocytes including $CD4^+$ T-helper (T_h) cells and $CD8^+$ T-cytotoxic (T_c) cells. Sahlander *et al* showed that T_h cells, especially IL-4 and IL-13 producing T_h2 cells, are more abundant in farmers and smokers than in non-farming non-smokers [24]. These cytokines (IL-4 and IL-13) are also known to stimulate MUC5AC protein expression [147] which increases mucus production important for developing chronic bronchitis [148].

Stimulated peripheral whole blood showed no significant differences of the percentage of T_c and T_h cells between budesonide and placebo treatments. The proportion of IL-4 and IL-13 expressing T_c cells decreased ($p=0.041$ and $p=0.016$ respectively, Figure 10) after budesonide treatment compare to before. Also placebo treatment significantly reduced the expression of IL-13 expressing T_c cells ($p=0.013$). Generally, the proportion of intracellular IL-13 and IL-4 producing T_c2 cells is: control<farmers<<smokers [24, 147]. As the bud-treated swine farmers significantly decreased T_c2 cells producing intracellular IL-13 and IL-4, the treatment possibly shift the T-cell profile of the farmers to resemble more of the healthy controls. In smokers with COPD, IL-13 is generally higher than in healthy controls [84] and *in vitro* IL-13 induces mucin/mucus production [43]. Both these observations indicate that IL-13 can be of importance in the development of chronic bronchitis and the bud-driven reduction of IL-13 and IL-4 could be an essential factor for reducing the risk of developing chronic bronchitis.

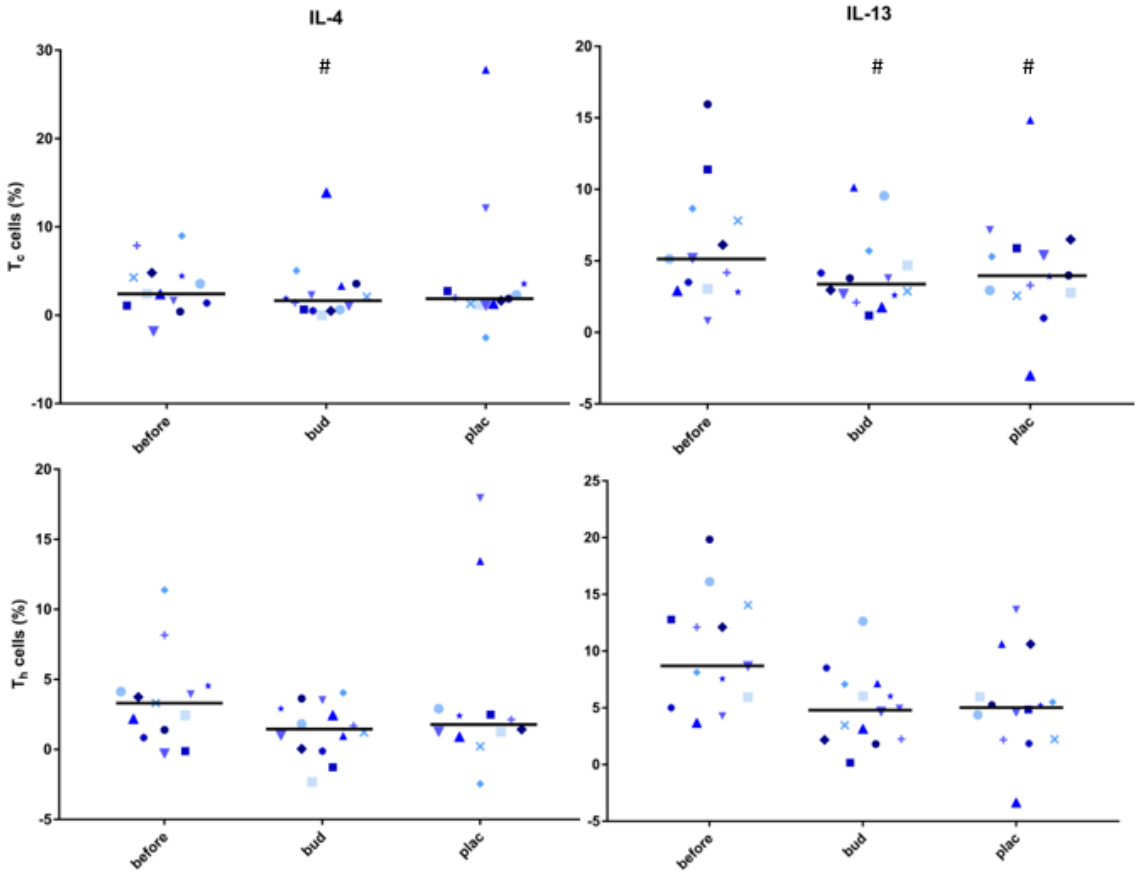


Figure 10. Percentage of stimulated peripheral T-helper (T_h) and T-cytotoxic (T_c) cells producing intracellular IL-13 and IL-4, before, after budesonide (bud), and after placebo (plac) treatment of swine farmers (n=14). Individual data and median (bold horizontal line) presented. # $p<0.05$ compared with before values.

In BALF (n=7) AM mRNA of 10 genes expressed during inflammation and innate immune responses were investigated before and after budesonide treatment directly after isolation. Expression of *GPx* mRNA in AM significantly increased (p=0.043) after *in vivo* treatment of the swine farmers compared to before treatment whereas the other genes were not affected by the treatment.

Ex vivo

Three comparisons were made to explore the mRNA expression of 5 genes (TLR2, TLR4, CD14, IL-13 and CXCL8) in the *ex vivo* stimulated AM, before and after budesonide (Figure 11):

1. Influence of *ex vivo* budesonide stimulation; comparing AM with and without budesonide for same TLR ligand stimulation.

2. Influence of *ex vivo* stimulation; stimulation compared to its own control (before and after respectively).

3. Influence of *in vivo* budesonide treatment; identical stimulation, compared before and after *in vivo* treatment (n=4-7).

Ex vivo stimulation of budesonide increased the *TLR2* mRNA expression before and after *in vivo*-treatment compared to non-stimulated control (both p=0.028). Expression of *TLR2* mRNA increased in AM after co-stimulation *ex vivo* with budesonide and peptidoglycan or LPS both before (PG; p=0.028, LPS; p=0.028) and after *in vivo* treatment (PG; p=0.028, LPS; p=0.046) compared to stimulation with LPS or peptidoglycan only. An increased expression was also observed when AM were co-stimulated *ex vivo* with budesonide and TNF-α (compared to TNF-α only), but only before *in vivo*-treatment (p=0.028, n=6). (Figure 11-1)

When comparing the influence of *in vivo* treatment on AM *TLR2* expression, no significant changes were observed.

Ex vivo co-stimulation of budesonide and LPS decreased mRNA AM expression of *CXCL8* both before (p=0.028) and after (p=0.028) *in vivo* treatment compared to stimulation with LPS alone. Both before and after treatment, the *ex vivo* LPS stimulated AM showed significantly higher (5 and 16 times respectively) expression of *CXCL8* mRNA than its own control (p=0.028 and p=0.028 respectively). Before *in vivo* treatment, *ex vivo* co-stimulation of AM with budesonide and TNF-α decreased mRNA *CXCL8* expression compared to stimulating with TNF-α alone (p=0.028). After *in vivo* treatment, *ex vivo* stimulation with peptidoglycan significantly increased (doubled) the *CXCL8* mRNA expression in AM from swine farmers compared to its own control (p=0.028). Co-stimulation of budesonide and peptidoglycan significantly decreased *CXCL8* mRNA AM expression in farmers after *in vivo* treatment compared to stimulating with peptidoglycan alone (p=0.028).

No clear *in vivo* effects of treatment on *ex vivo CXCL8* mRNA expression were detected when comparing before and after *in vivo* treatment in AM (Figure 11-3).

Ex vivo co-stimulation of bud and peptidoglycan significantly reduced CXCL8 release by AM of farmers before *in vivo* treatment compared to peptidoglycan alone (p=0.028, n=6). None of the other (co-)stimulations significantly altered the CXCL8 release. Farmers treated *in vivo* increased CXCL8 concentrations in *ex vivo* budesonide and peptidoglycan + budesonide stimulated AM compared to comparable stimulations of non-treated farmers (p=0.028 and 0.046 respectively) (n=6). The *in vivo* treatment diminished the reduction of CXCL8 observed pre-treatment when *ex vivo* budesonide was added to peptidoglycan stimulated AM, described above (Figure 11-3).

In commercially available normal human epithelial cells, the alveolar cell line A549 and AM from healthy controls, swine dust dose-dependently increases CXCL8 production [149] whereas budesonide causes dose-dependent inhibition of CXCL8 production in AM and increased *TLR2* mRNA expression [150]. von Scheele *at al*, 2010 showed similar trends in *TLR2* mRNA upregulation and CXCL8 reduction upon steroid and ligand stimulation [146]. *In vitro* co-stimulation of primary bronchial epithelial cells showed reduced *CXCL8* mRNA expression and CXCL8 secretion where swine dust + budesonide gave a larger reduction than LPS + budesonide and also than the TLR ligand only. Generally this study also show a decrease in CXCL8 expression and release when being co-stimulated. A different trend could be observed in this study: the CXCL8 release especially and somewhat the mRNA expression of *CXCL8* are increased after *in vivo* treatment compared to control. In two of the farmers, CXCL8 increased in all *ex vivo* stimulated AM after *in vivo* treatment compared to before. This increase

could be explained by the increased mRNA expression of *TLR2* that was revealed in the *ex vivo* experiment. The more available pattern recognition receptors the more pathogen-associated molecular patterns in organic dust could be bound, which could result in increased expression and release of CXCL8.

It has been suggested that the formation of sTLR2 (shedding) is a cellular response to reduce over-stimulation of the cell surface receptor occurring in smokers with COPD [65]. In this study, release of sTLR2 from AM was below detection limit, except for two *in vivo* treated farmers where detectable concentrations of sTLR2 were measured in LPS and peptidoglycan ± budesonide stimulated AM. As sputum sTLR2 significantly increased post budesonide *in vivo* treatment shedding activities is likely also in farmers.

In vivo treatment and *ex vivo* stimulations of AM with budesonide or TLR ligands did not alter *TLR4* mRNA expression, except in one case; after *in vivo* treatment, the stimulation of peptidoglycan significantly decreased (by half) the *TLR4* expression compared to its own after treatment control (p=0.028, n=5) (Figure 11-1).

Ex vivo stimulations with LPS, peptidoglycan or TNF-α did not alter *CD14* mRNA expression in AM (Figure 11-2).

Budesonide stimulation *ex vivo* of AM from *in vivo* treated farmers significantly lowered the expression of *CD14* mRNA compared to its own control (p=0.028, n=6). Also, *CD14* mRNA expression more than doubled in the control after *in vivo* treatment compared to before (p=0.043, n=5)(Figure 11-2). Increased *TLR2* and *CD14* mRNA expression implies a higher availability of receptors for ligands, leading to a pro-inflammatory response including the increased concentrations of CXCL8 in AM.

Concentrations of secretion of sCD14 and IL-13 in AM were below detection limits (62.5 and 93.8 pg/ml respectively) (n=6).

Before treatment, e*x vivo* co-stimulation of budesonide and TNF-α significantly decreased the mRNA expression of *IL13* compared to TNF-α alone (p=0.028, n=6)(Figure 11-2).

In vivo effects of budesonide were observed for *IL13* mRNA expression. In the non-stimulated controls, the mRNA expression of *IL13* significantly increased in *in vivo* treated farmers compared to before treatment (p=0.043). After *in vivo* treatment, *IL13* mRNA expression in AM increased when being co-stimulated with budesonide and peptidoglycan compared to the same stimulation before *in vivo* treatment (p=0.043, n=5)(Figure 11-2).

In this study, we used two different models to analyse mRNA expression in AM. One where the AM were directly prepared from BALF and the other after over-night starvation without added serum to the culture media followed by a six hours incubation with different stimuli. Analysing 10 genes using direct preparation only one (*GPx*) was significantly increased after *in vivo* treatment. After starvation, five genes were examined and *IL-13* and *CD14* were significantly increased after budesonide *in vivo* treatment of farmers. Over-night starvation of AM seems to be required to synchronise cells to achieve measureable non-confounded levels of mRNA.

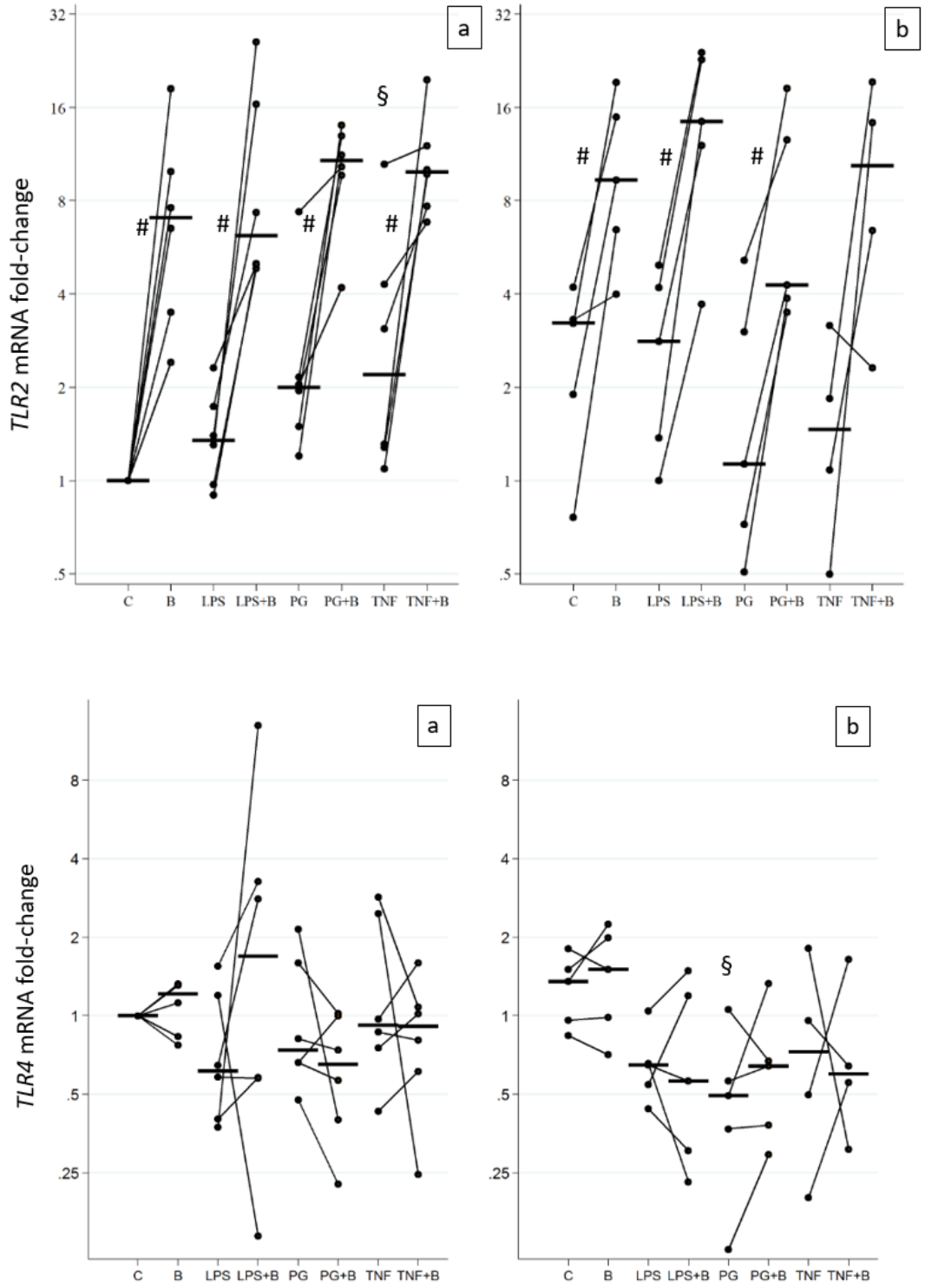


Figure 11-1. Expression of *TLR2* and *TLR4* mRNA from AM isolated from swine farmers' bronchoalveolar lavage fluid, before (a) and after (b) *in vivo* budesonide treatment, incubated for 6 hours in control medium, LPS (lipopolysaccharide), PG (peptidoglycan) or TNF-α with or without budesonide (B) including medians (bold line). # $p<0.05$, stimulated cells; with B compared to same stimulation without B. § $p<0.05$ stimulated cells; stimulation without B compared to its own control (before or after) *$p<0.05$, identical stimulation, compared before and after *in vivo* treatment (n=4-7).

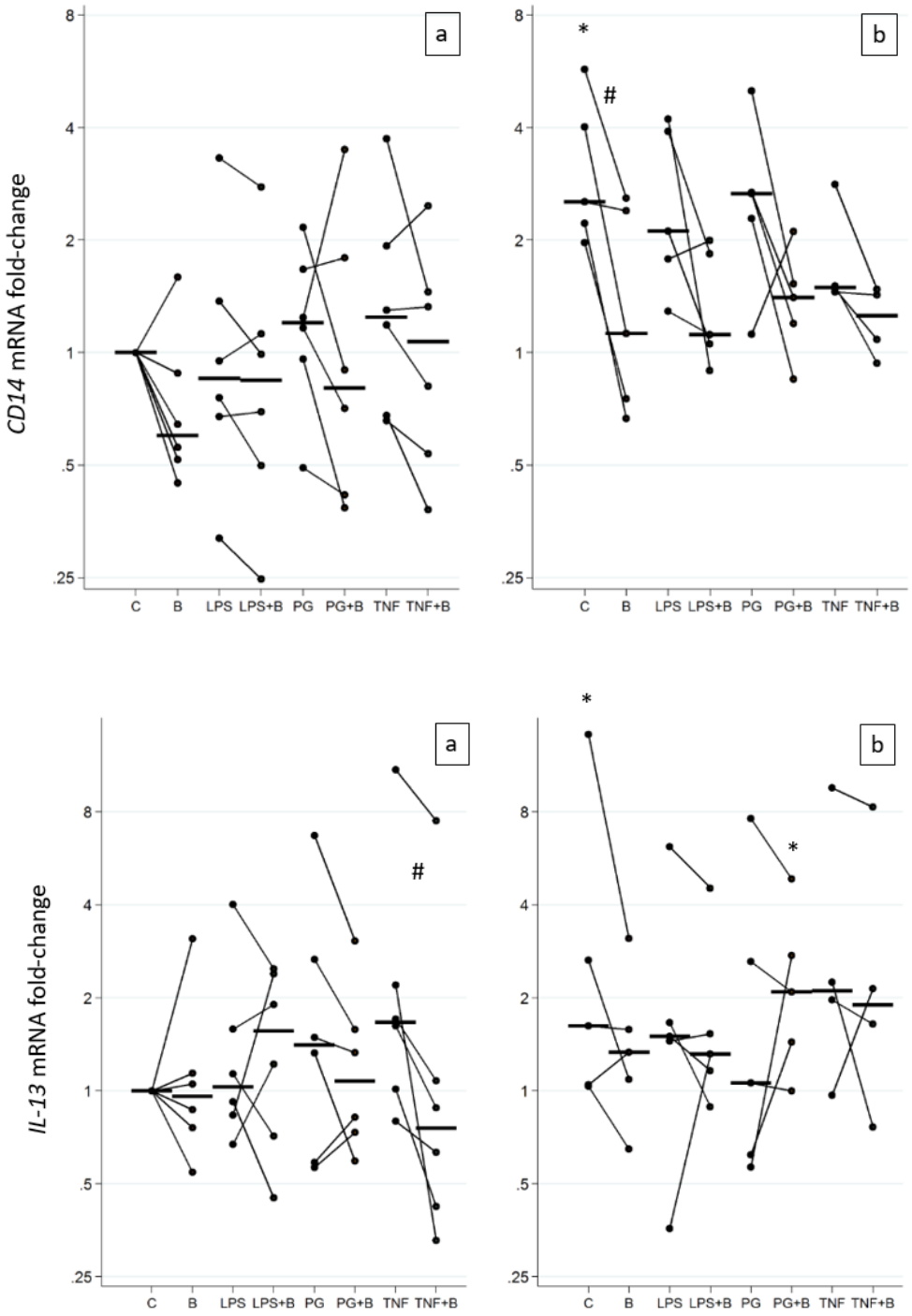


Figure 11-2. Expression of *CD14* and *IL13* mRNA from AM isolated from swine farmers' bronchoalveolar lavage fluid, before (a) and after (b) *in vivo* budesonide treatment, incubated for 6 hours in control medium, LPS (lipopolysaccharide), PG (peptidoglycan) or TNF-α with or without budesonide (B) including medians (bold line). # p<0.05, stimulated cells; with B compared to same stimulation without B. § p<0.05 stimulated cells; stimulation without B compared to its own control (before or after) *p<0.05, identical stimulation, compared before and after *in vivo* treatment (n=4-7).

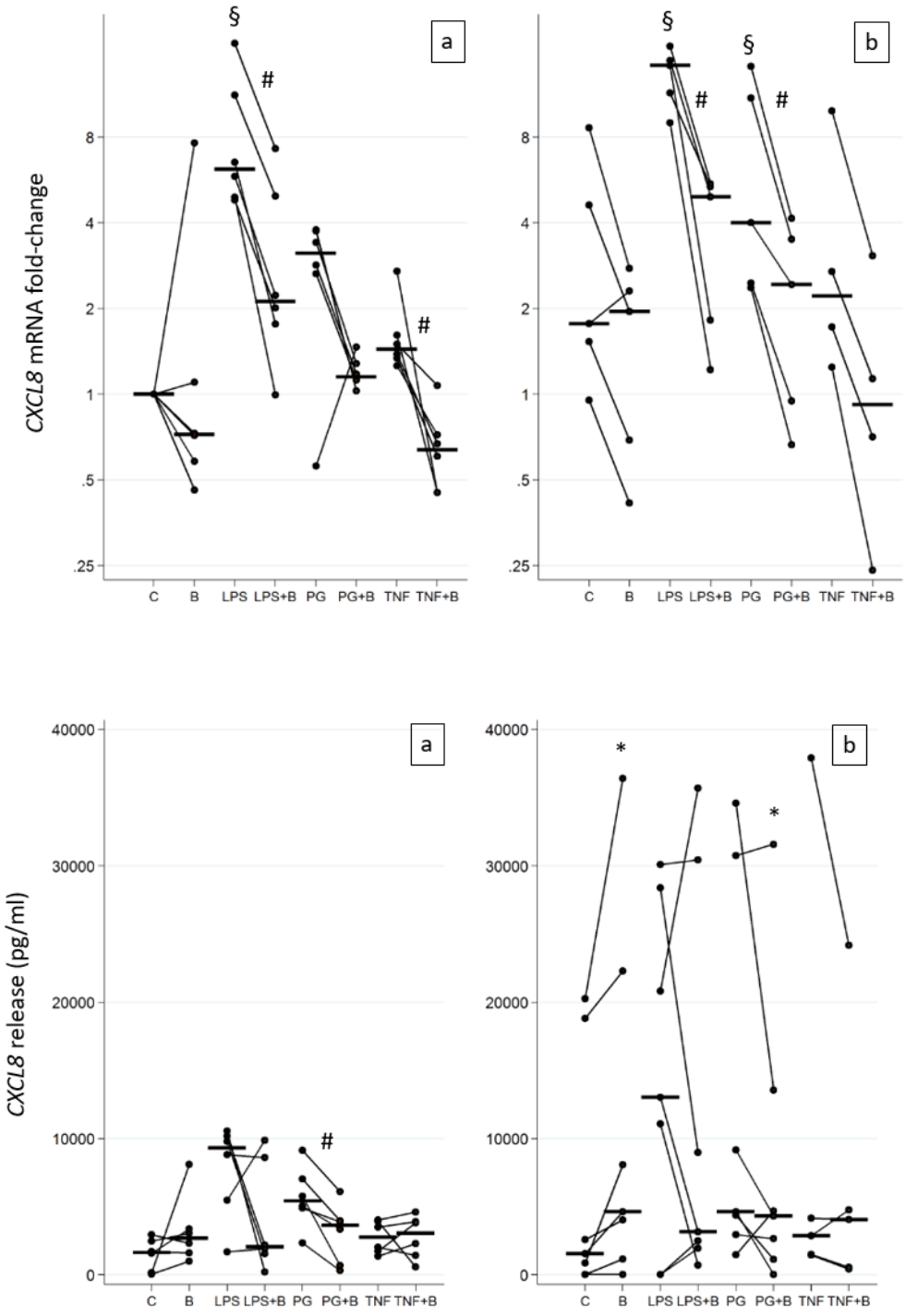


Figure 11-3. Expression of *CXCL8* mRNA and release of CXCL8 from AM isolated from swine farmers' bronchoalveolar lavage fluid, before (a) and after (b) *in vivo* budesonide treatment, incubated for 6 hours in control medium, LPS (lipopolysaccharide), PG (peptidoglycan) or TNF-α with or without budesonide (B) including medians (bold line). # $p<0.05$, stimulated cells; with B compared to same stimulation without B. § $p<0.05$ stimulated cells; stimulation without B compared to its own control (before or after) *$p<0.05$, identical stimulation, compared before and after *in vivo* treatment (n=4-7).

Correlations

To elucidate the possible interactions between some of the measure endpoints, correlations analysis were made (Figure 12). Some obvious correlation clusters of lung function and blood cell distribution were found but also some more unforeseen.

When investigating lung function and number of blood cells, it is clear that the more impaired lung function (VC, FEV_1 and FVC) the more leucocytes in peripheral blood (lymphocytes, neutrophils and eosinophils, $p \leq 0.03$). When adjusting lung function for height and age, all lung function parameters were positively correlated to both sCD14 and sTLR2 in BALF ($p \leq 0.005$).

Soluble TLR2 in BALF correlated with sCD14 in BALF (p=0.005), but also sST2 in serum (p=0.005). Whereas sST2 in serum correlated to sCD14 in BALF (p=0.04) and to both TLR2 and CD14 on blood monocytes (p=0.05 and 0.009). All correlations were positive. The co-variation between serum sST2 and sTLR and sCD14 in BALF have never previously been reported. These correlations are observed even if there are no significant treatment-related changes for these endpoints. It suggests that less invasive methods like measuring sST2 in blood could be a relevant and a useful biomarker for measuring host innate defense instead of investigating the more known soluble proteins sTLR2 and sCD14 in bronchoalveolar lavage.

TLR2 on blood monocytes were positively correlated to CD14 expression on both monocytes and neutrophils (p=0.0001 and 0.030 respectively), whereas negatively correlated to TLR2 and TLR4 on neutrophils (p=0.0005 and 0.002). TLR2 on neutrophils were positively correlated with TLR4 expression on neutrophils and lymphocytes (p= 0.02 and 0.02) but negatively correlated to CD14 expression on monocytes (p=0.01). TLR4 expression on neutrophils were positively correlated to TLR4 on lymphocytes ($p=6.9\times10^{-5}$). TLR4 expression on neutrophils was also negatively correlated to CD14 expression on monocytes and neutrophils (p=0.04 and 0.03). Interestingly, Sahlander *el at*, 2012 found that farmer TLR4 on blood neutrophils negatively correlated to soluble CD14 in sputum [81].

Correlations on directly prepared AM mRNA showed that *TLR2* correlated to *TIMP* (p=0.02), *SLPI* to *SOD3* and *GPx* (p=0.05 and 0.02 respectively), *IL13* to *SOD3* and *LL37* (p=0.05) and 0.02 respectively) and *SOD3* to *LL37* (p=0.04). *GPx* also correlated to TLR2 expression on blood neutrophils and lymphocytes. All were positively correlated.

LeVan *et al*, 2005 showed that the CD14/-159 and CD14/-1619 loci positively associated with wheezing in farmers [151]. We also found a correlation between wheezing and by mRNA *CD14* expression on directly prepared AM (p=0.034). mRNA expression of *CD14* in AM was positively correlated to sCD14 in serum (p=0.01) whereas sCD14 in BALF correlated to shortness of breath (p=0.042).

mRNA expression of *TLR2* in AM was negatively correlated to sTLR2 in serum (p=0.01) and *TLR4* in AM was negatively correlated to sTLR2 in sputum (p=0.03).

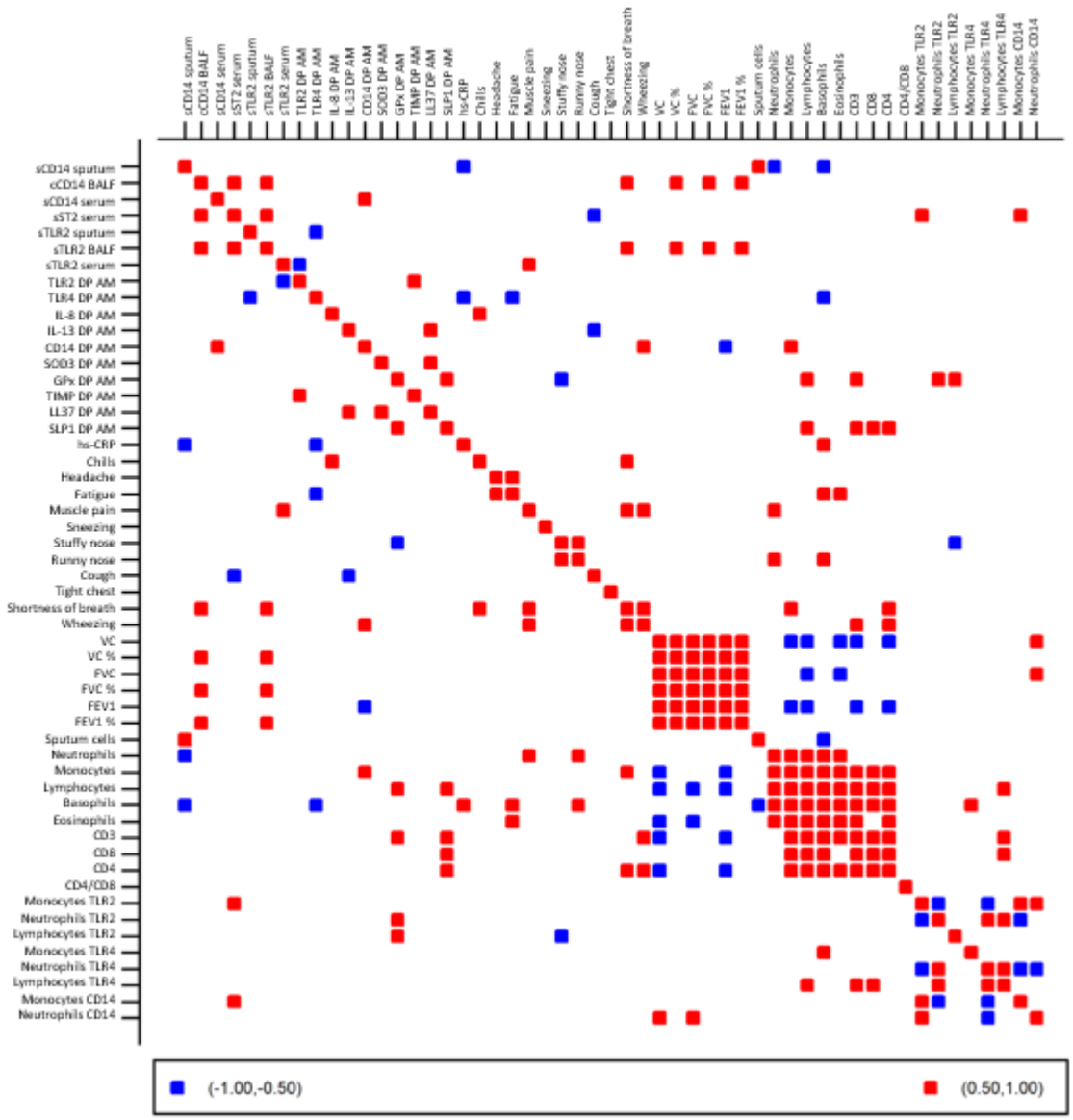


Figure 12. Heat-map of Spearman rank correlations of data-points from non-treated swine farmers. Blue square; negative correlation (ρ=-0.50- -1.00), p<0.05 Red square; positive correlation (ρ=0.50-1.00), p<0.05.

As in all complex study models there are drawbacks with the systems used. Here, the short two-week treatment period, the limited number of available swine farmers in commuting distance to Stockholm area combined with the farmers phenotypic diversity could likely have influenced the results of the study. Polymorphism of the farmers' TLR and glucocorticoid receptors have not been investigated in this study. Even if performing a crossover design study, which generally reduces the between-subject variability and increases power by limiting non-treatment related factors, the above limitation cannot be fully overcome. The healthy worker effect is another possible limitation, as farmers with pulmonary problems may have stopped working at the swine farm.

In conclusion, we found in this crossover study that swine farmers, who are chronically exposed to organic dust who inhale glucocorticosteroid for two weeks increase the release of soluble

TLR2 in the airways and the number of circulating leucocytes. The TLR4 expression on lymphocytes increased and the cytotoxic T-cell surface expression of IL-13 and IL-4 decreased after Bud-treatment. Additionally, mRNA expression in AM from a smaller subset of farmers increased *TLR2* and decreased *CXCL8* after *ex vivo* co-stimulation with LPS/peptidoglycan/TNF-α and Bud. The mRNA expression of *CD14*, *IL-13* and *GPx* in AM increased after the *in vivo* Bud-treatment of swine farmers.

These *in vivo* findings confirm recent *in vitro* results using epithelial cells and macrophages from healthy donors; down-regulated TLR2 in swine farmers' immune cells increase after short-term treatment with ICS. Our findings indicate that inhalation of a glucocorticosteroid strengthens immune defense pathways in subjects with occupational chronic exposure to organic dust. This may have implications for understanding the steroid effect in the treatment of conditions such as chronic bronchitis and COPD that are characterized by microbial colonisation in airway conditions.

PAPER III

The aim of this paper was to first develop an advanced three-dimensional *in vitro* system resembling a bronchial airway mucosa and second to combine this model with a complex exposure technique to mimic the *in vivo* scenario.

Human primary bronchial epithelial cells (PBEC) formed the basis of the model in the insert together with fibroblast MRC-5 cell line grown under the insert membrane facing the well. Initially the cells were grown in collagen [152, 153] but as the models shrunk, the exposure dose of the models would have been difficult to calculate. The co-culture of these cells were maintained under air-liquid interphase where medium was only supplied in the well under the insert. Once a stable model was developed, research to establish a chronic bronchitis-like models was initiated. By adding IL-13 at the start of air-lifting, the modified mucus producing model was developed.

Palladium is a rare metal found in the upper crust of earth and is commonly used in nanoform in catalytic converters in the automobile industry. Palladium nanoparticles is present in air as airborne automotive pollution caused by abrasion of catalyst material of the car exhaust system. Previously, toxicity of palladium particles was assessed *in vitro*, using PBEC and alveolar cell line A549 and cultured and exposed under submerged conditions [130]. Using the newly developed Xpose*ALI*®/PreciseInhale, the new bronchial models could be exposed to aerosolized nano-sized palladium particles.

Beating of ciliated cells including the typical microtubules structure of the cilia, differentiation into goblet cells and club cells are important characteristics of the airway mucosa. All these requirements were important when establishing a mucosa model. For chronic bronchitis, the hyper/metaplasia of mucus producing cells was crucial.

Evaluating the models using different staining techniques, it was clear that all important features for a bronchial mucosa were achieved. Already in light microscope beating cilia was seen. H&E staining revealed the differentiation into basal cells, club cells, mucus producing cells and ciliated cells (Figure 13A). Immunofluorescent staining and confocal microscopy confirmed cilia (in green) and expression of MUC5AC (red) on goblet cells shown in Figure 13B. To confirm cilia containing axonemes with two central singlet and nine outer doublet microtubules, SEM (Figure 13C) and TEM (Figure 13D and E) were required. Figure 13E also confirms the presence of electro-dense cytoplasm typical for mucus producing cells.

To assess the differentiation of the cells, mRNA expression of specific cell markers was studies over a three-week period. Only *FOXJ1* mRNA expression, a ciliated cell marker, significantly increased over time when being air-lifted as detected by RT-PCR (Figure 14A). MUC5AC, club cells protein and KRT5 (basal cell marker) did not alter mRNA expression being airlifted for three weeks (Figure 14B, C and D).

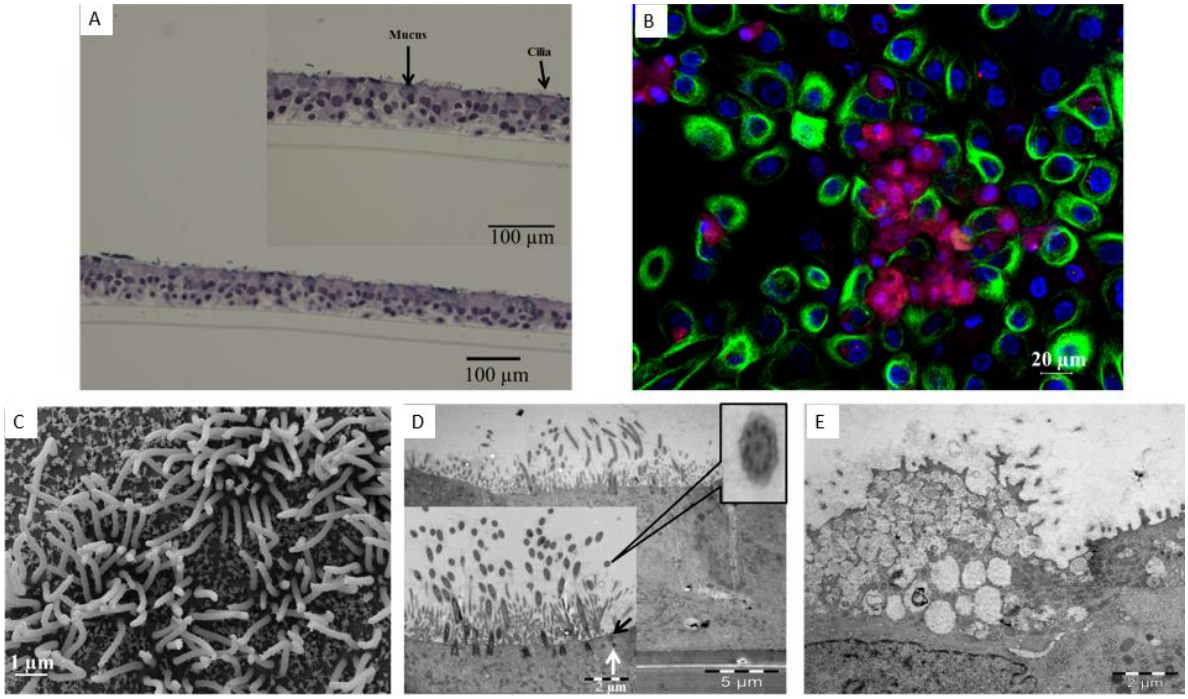


Figure 13. Morphological characterization of 3D model [45].

A: Light microscope analysis of hematoxylin and eosin (H&E) staining of paraffin embedded cross section of 2 weeks ALI model; Bar scale: 100 µm. Higher magnification showed cilia and mucus; Bar scale: 100 µm.
B: Confocal microscope analysis of immunofluorescence staining of ciliated cell marker anti-acetylated alpha tubulin antibody (green florescence), mucus producing cell marker anti-MUC5AC antibody (red florescence) in 2 weeks ALI model; cell nuclei stained with DAPI (blue florescence). Bar scale: 20 µm.
C: Scanning electron microscope analysis of 2 weeks ALI model, mature cilia present; Bar scale: 1 µm.
D: Transmission electron microscope analysis of 2 weeks ALI model, ciliated cell surface was scattered with elongated cilia; Bar scale: 5 µm. Higher magnification showed cilia displaying 9+2 axoneme formation, tight junction (black arrow) and desmosome (white arrow); Bar scale: 2 µm.
E: Transmission electron microscope analysis of 2 weeks ALI model, mucus cell present with electron-dense cytoplasm containing electron-lucent granules; Bar scale: 2 µm.

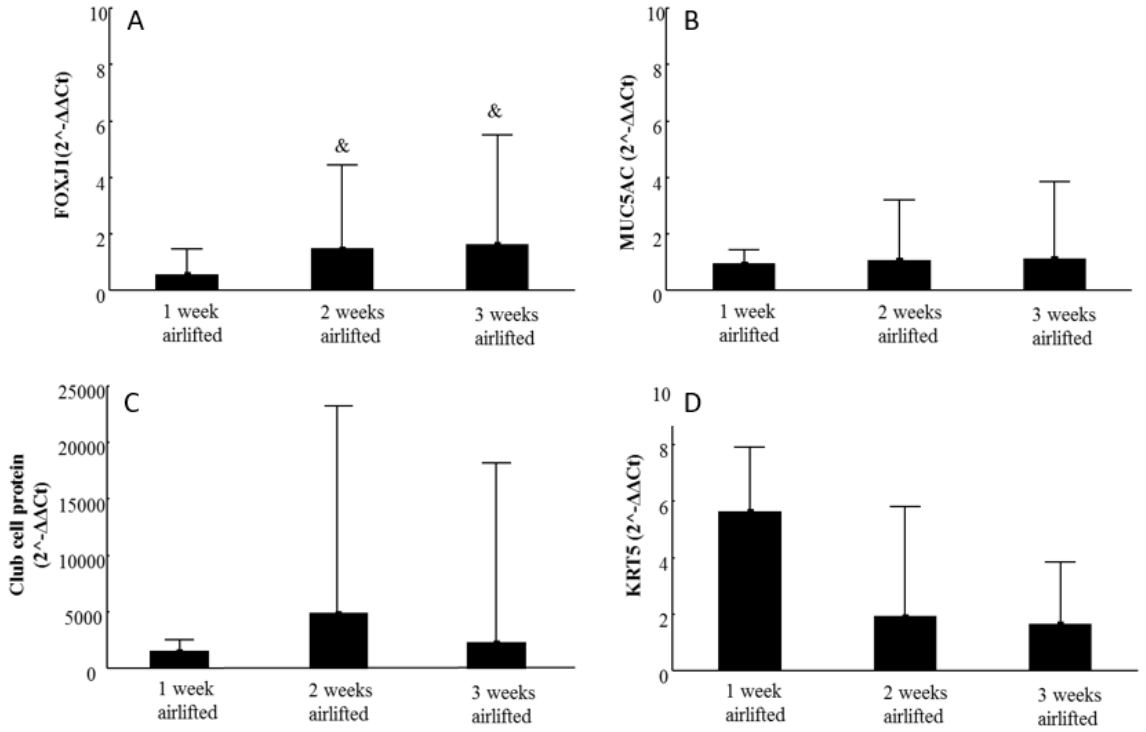


Figure 14. mRNA expression of different cell type markers in 3D models. Expression of ciliated cell marker *FOXJ1* (A), mucus producing cell marker *MUC5AC* (B), club cell marker Club cell protein (C) and basal cell marker *KRT5* (D) mRNA in normal models after culturing at ALI for 1, 2 and 3 weeks (N = 9). Data presented as median and 25th -75th percentiles; &: P<0.05 VS *FOXJ1* mRNA expression in 1 week culturing at ALI [45].

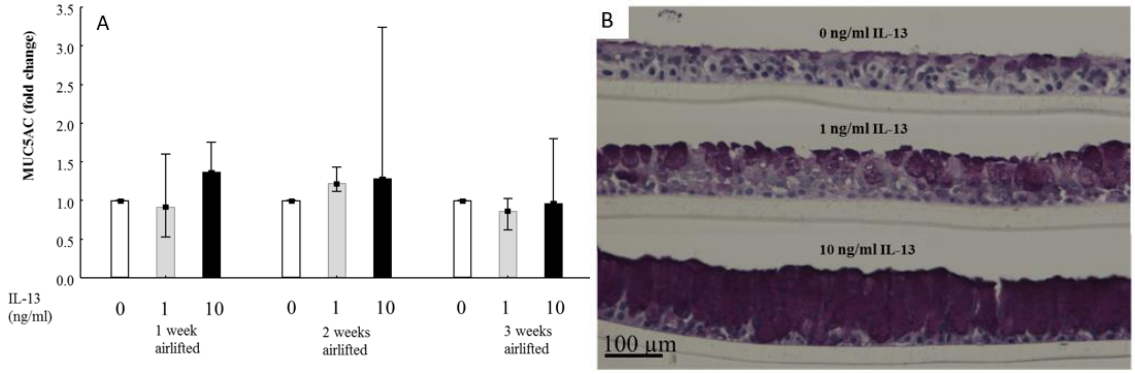


Fig 15. Effects of IL-13 stimulation on 3D models. A: Fold change of MUC5ACmRNA expression in ALI models treated without (blank) and with 1 ng/ml (grey) and 10 ng/ml (black) IL-13 for 1, 2 and 3 weeks (N = 9); Data presented as median and 25th -75th percentiles. B: Periodic acid—Schiff (PAS) staining of paraffin embedded cross section of ALI models treated without and with 1 ng/ml and 10 ng/ml IL-13 for 2 weeks visualized by light microscope; Bar scale: 100 µm. [45]

When treating the model with 10 ng/ml IL-13, almost all cells differentiated into mucus producing cells as shown by PAS staining in Figure 15. Instead, a lower concentration of 1 mg/ml IL-13 was chosen to modify the mucosa model into a chronic bronchitis-like model.

To validate the viability of the models trypan blue showed that >95% viable cells and the apoptotic rate 1-30%. TEER showed no difference in integrity of the barriers and tight junctions between the normal and the chronic bronchitis-like model. The models were kept viable for 3-4 weeks after being air-lifted. The first sign of model function loss is the cilia that reduce in number as well in structure, soon becoming bud-like.

Second step was to introduce the palladium particles and the aerosol exposure system. X-ray diffraction and EDS analysis of the palladium particles showed high purity. Palladium in ethanol solution [130]) was vacuum dried and yet the particles agglomerated during this process, they easily aerosolized in small particles (6-10 nm) when shot in PreciseInhale™ (Figure 16).

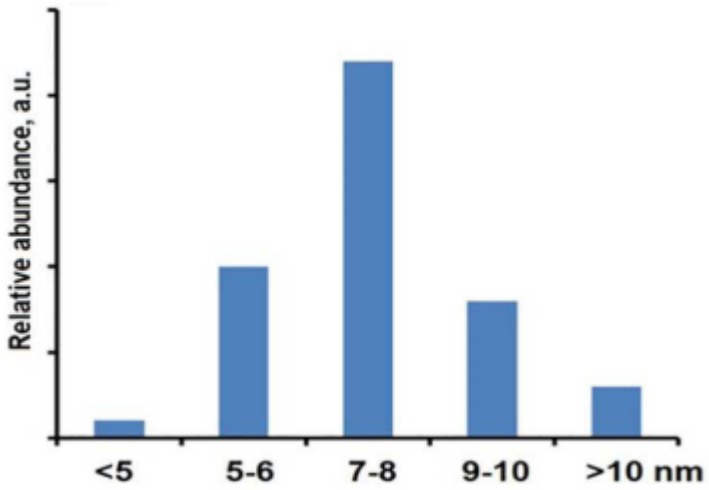


Figure 16. Size distribution of aerosolized Pd nanoparticles. Size 6–10 nm with a distinct maximum at 8 nm [45].

Using PreciseInhale™ and Xpose*ALI*® for palladium exposure showed evenly distributed particles throughout the insert and the cells. Most of the particles stayed on top of the cells whereas some internalized in the cells and longer incubation time seemed not to influence this finding. After 24 hours post-exposure the mucociliary transport of the models seem to have relocalized the palladium to one corner of the insert (Figure 17). This is very different than dispered palladium nanaoparticles in submergely cultivated PBEC [130]. Within two hours, PBEC had taken up the majority of palladium particles now found in membrane-bound vesicles, likley due to active uptake (endocytosis) [154, 155]. Not only are the exposure settings very different from using ALI conditions and aersol exposure, but also is it likely that the nanoparticles in media is surronded by a corona of biomolecules that could affetcs the toxicity

of the particle and modulating the particle recognition by the cell. [156]. As the mucos surfacing the cilia in the models likely protects the cells from actual exposure to nanoparticles, the *in vitro* 3D model much better represent the real life situation.

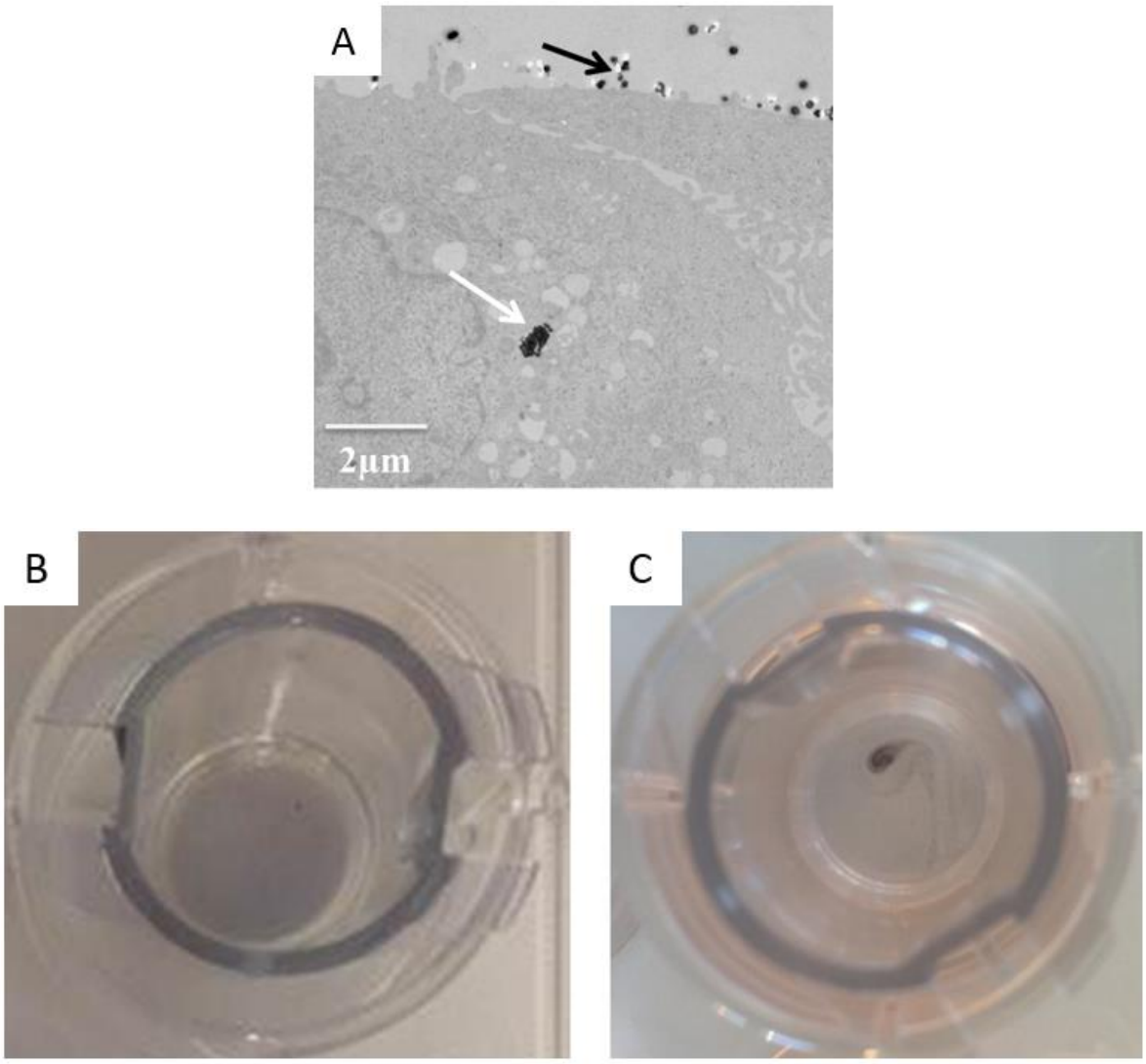


Figure 17. Uptake and clearance of palladium nanoparticles *in vitro*. A: The internalization of palladium nanoparticles in bronchial mucosa model, incubated 24 hours post-exposure. Most palladium nanoparticles are localized on the cell surface (black arrow) and few nanoparticles are localized inside the cells (white arrow). Bar scale: 2 µm. Mucociliary clearance: B: Even distribution of palladium nano-particles (high dose: 650 ng/cm^2). C: Relocalization of palladium nanoparticle. Accumulated in one spot within 24 hours, indicating mucociliary clearance. [45]

After exposure, viability was assessed (as above) and exposure did not affect the viability in any of the models.

Wilkinson *et al*, 2011 [130] demonstrated that dispersed palladium nanoparticles in submergely cultured PBEC increased CXCL8-release to the culture media. In our study, the release of CXCL8, MMP-9 and club cells protein in apical and basal medium of both models (+/- IL-13) were measured at 8 and 24 hours post palladium aerosol exposure (control, 250, 400 and 650 ng/cm^2). The concentration of club cell protein was not affected by neither IL-13 nor palladium exposure. Neither the release of MMP-9, important for remodeling of lung tissue and mainly secreted by cells upon inflammatory stimulation [85, 157], was altered by IL-13 stimulation or palladium nanoparticle aerosol exposure. Much higher concentrations of MMP-9 was found in apical media than in basal media, even if the apical media was only collected for 15 minutes by lavage whereas the basal media contained the cumulative release of MMP-9 over 8 or 24 hours. For CXCL8, this apical/basal media difference was not observed (Figure 18). Upon palladium exposure, CXCL8 release was higher (significantly after 24 hours and similar trend after 8 hours post-exposure) in the chronic bronchitis-like models than in the normal models. If the smaller CXCL8 (8.4 kDa) was mainly produced on the apical side, it could more easily penetrate the confluent layer of differentiated bronchial cells and the membrane pores than the slighter larger MMP (92 kDa). The pores are likely not the limiting factor as they are sized 400 nm in diameter. The differences in molecular weight of the proteins is less obvious when protein size is estimated as a10 kDa protein is about 3 nm in diameter and 100 kDa protein is about 6 nm [158]. This suggests that MMP-9 is possibly secreted by epithelial cells, whereas CXCL8 is probably produced by both epithelial and fibroblasts.

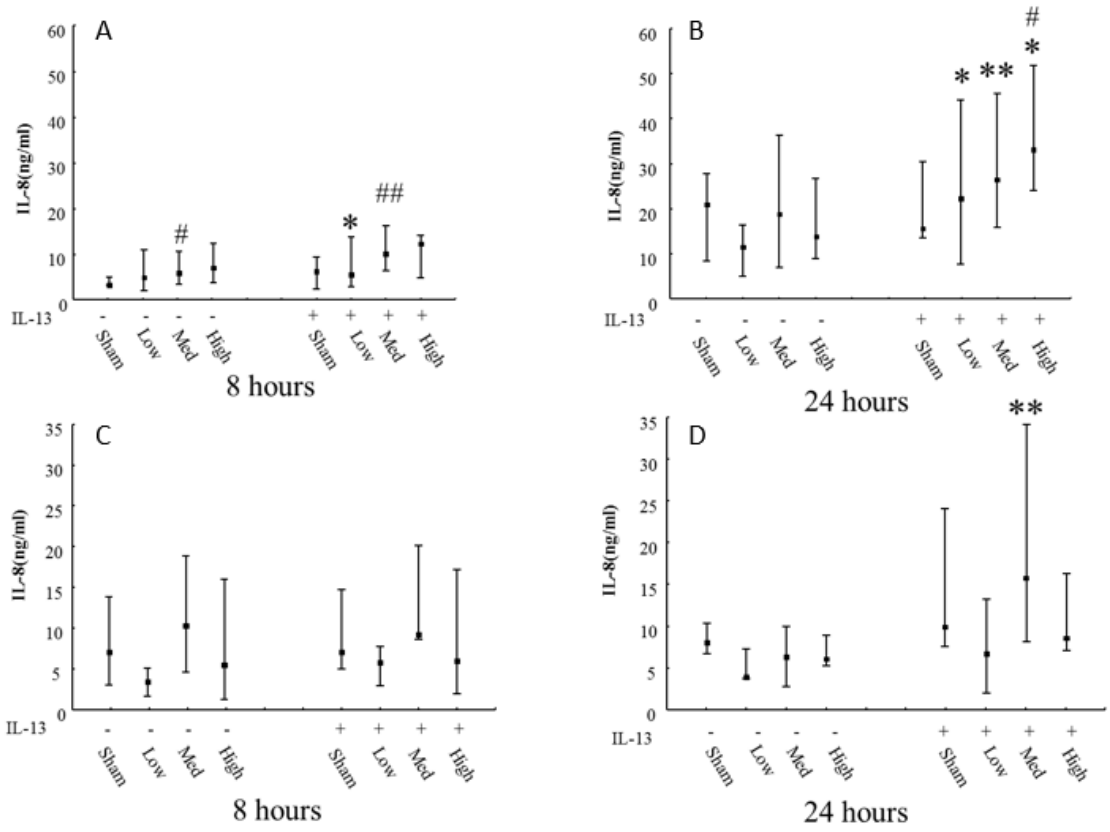


Fig 18. Release of CXCL8 (IL-8) after palladium nanoparticle exposure using Xpose*ALI*®.

Post-exposure concentrations (8 or 24 hours) of CXCL8 in basal (A and B) and apical (C and D) media of normal or chronic bronchitis-like (1 ng/ml IL-13) models (n = 9) models exposed to palladium nanoparticles (sham: normal air and clean system; low: 250 ng/cm^2; med: 400 ng/cm^2; high: 650 ng/cm^2). Median presented with 25th - 75th percentiles; #: $p<0.05$ vs sham exposure; *, **: $p<0.05$, 0.01 vs normal model. [45]

GENERAL DISCUSSION

Todays' global consumption of meat and mainly pork have led to intense animal production with high through-put in large scale [31, 159]. According to statistics from Swedish Board of Agriculture, the Swedish meat consumption doubled from 1960 till today (from almost 25 kg to 50 kg per person and year). In the last 10 years, there has been a slight decrease probably due to today's focus on environmental concerns linked to meat production.

Swine intended for meat production live their whole life (about 0.5 year) inside an enclosed swine building, while farmer can be found working in this environment 40 hours per week for more than 40 years. Exposure to organic swine dust is an important health risk to consider for both the farmers working in the barns as well as housed swine. Organic dust is not exclusive to swine farming but is also present in other agricultural settings (poultry, grain) as well as the textile (cotton) and the forest (wood) industries. The organic dust contains pro-inflammatory bacterial fragments like endotoxin and peptidoglycan. Inhaling particles and these constituents lead to an increased risk of reduced lung function [160, 161] and developing respiratory diseases like chronic bronchitis and COPD [162-164].

One of the basic principle of risk management in occupational hygiene is the hierarchy of controls including elimination, substitution, engineering and administrative controls and lastly the use of personal protective equipment [165]. The concentration of organic dust can be high in conventional swine production but few studies have investigated if the dust levels can be reduced by organic production. In 2007, airborne concentrations and composition of particles was compared in conventional and organic swine production [166]. The German study included two organic farms complying with European directive on organic production of agricultural products (EEC 2092/91, 2003) where the main difference is the requirement of using bedding material. Here, no difference in indoor organic dust and endotoxin levels was shown. Yet it is unclear if or how long these swine were outdoors. Swedish animal production conditions for both conventional and organic swine are in many areas more stringent than the rest of the Europe and the world. Swedish conventional production is more protective of the swine and the environment including less use of antibiotics, bedding requirement and minimum space allowance per swine. Swedish organic swine, according to KRAV®, need to be outdoors at least 4 months per year. This suggests that organic production causes less exposure to organic swine dust of both swine and workers but studies show that this production system could introduce other occupational risks [167].

In this thesis, the first paper focused on the use of engineering controls to reduce the exposure to the highly potent organic dust by assessing the efficiency of cyclones to reduce dust and fine particles in swine barn air and the health impact of exposure volunteers. The cyclones reduced the fine particles in the air and both local and systemic adverse effects in the volunteers diminished.

The study was performed during the summer and both out-door temperature and humidity fluctuated during the study period, although it was performed within a month in early summer.

Generally these parameters affect particle formation and duration in air and it is well-known that particle concentration in swine buildings are higher during winter than in summer [31, 141]. During summer the ventilation is high to maintain a good climate (temperature) for the swine which are very sensitive to heat [168]. The ventilation reduces the particles in the air by high air-exchange and hot pigs are very inactivity which also reduces the particle numbers in air. The installed cyclones ran during these conditions which were unfavourable to the study in order to understand their actual efficiency and effectiveness. To better assess this, future studies should be performed during winter when less ventilation and higher particle concentrations are likely. Combined with good house-keeping and cleaning routines including high-pressure hosing between the pig batches the cyclones could possibly be a viable and effective measure to reduce exposure to polluted air.

Reducing exposure of swine dust is important not only to reduce immunological alteration of the farmers. Over the last years, swine dust containing live-stock methicillin-resistant *Staphylococcus aureus* (LA-MRSA) have highly increased [169, 170]. Notably in Denmark, the prevalence of infected farms has gone from 16% in 2010 to 88% in 2016. The presence of antibiotic resistance is widespread in Europe and globally and both country and production type seem to be important factors for the occurrence [171, 172]. Infections of LA-MRSA impact not only swine, but also the farmers, the farmer's families and swine farm veterinarians. The bacterial colonisation in the airways of swine farmers might be important for the development of swine dust tolerance and is associated with increased frequency of COPD exacerbations [173]

The tolerance to organic swine dust appears in current and former farmers [101, 102, 174]. The tolerance development itself might not be beneficial to the farmers in the long-term perspective if it is related to their known increased risk of developing chronic bronchitis and COPS [160]. Still there is lack of understanding of the mechanism behind the tolerance [175]. To reduce the risk of tolerance development and immune system alteration, many methods to reduce dust exposure have been tried over the years, including robot cleaning and oil spraying. Up-regulating the host response in farmers has to date never been tried. This is why we in paper II explored the influence of budesonide treatment of current healthy swine farmers. The crossover study with budesonide and placebo resulted in increased sTLR2 in sputum and TLR4 on lymphocytes and decreased T_c cells producing IL-4 and IL-13 after budesonide treatment. Also, *CD14*, *IL-13* and *GPx* mRNA expression in alveolar macrophages increased after the *in vivo* steroid treatment. In all, the results indicate that short-term (2 weeks) treatment with inhaled budesonide up-regulates the down-regulated immune defense observed in chronically exposed swine farmers. However, it is unclear if long-term treatment would have the same effects or if there are any unwanted side effects. Short-term the steroid treatment boosted the immune system of the farmers, which could likely be sustained by prolonged treatment. Yet, it is unclear if this would be beneficial for the farmers long-term. The tolerance to swine dust is the host's adaptation to the environment and such treatment and alteration of the immune system makes the farmer's immune system resemble that of healthy volunteers who develop ODTS once exposed to the swine farm environment. It is obvious that treating farmers so that they

subsequently develop ODTS would not be a desired outcome. Long-term ICS is used for asthma- and COPD treatment [176]. Reported adverse effects from long-term use include growth suppression in children and reduced bone density in elderly. Since farmers do not belong to these patient groups it is difficult to speculate on the possible adverse long-term effects.

In both paper I and II we used human *in vivo* experiments to assess the short- and long-term influence of the swine dust. Instead of using a human model, swine model could be a useful alternative since the animal is anatomically and physiologically similar to humans [177]. It has even been suggested that the swine themselves could be used for assessing the risk of occupational hazard of farm workers [159].

Instead of swine as an alternative to human exposure experiments, we used human (primary) cells in paper III. By combining a species-relevant organotypic model with actual air pollutants we created an *in vitro* model resembling an *in vivo* situation. Throughout the development there has been a lot of "learning by doing" and thereby continuous technical and biological/cellular improvements including choice of media and cells, cell co-culture settings and culture techniques. This *in vitro* journey has just started and there are still many possibilities for further developments. For instance, long-term and/or chronic experiments and possibly even alveolar models could be developed for even further refined and accurate responses to ultrafine and ambient particles in air.

Additionally, the existing bronchial model could be altered and explored in many ways e.g origin of epithelial donors, new unstudied substances or mixtures or perhaps even new co-culture combinations. To study the epithelial difference from different groups of donors one example could be groups of different alcohol consumption including farmers. Alcohol consumption is known to increase risk of bronchitis and *in vitro* impair TLR2 on airway epithelial cells and their mucociliary clearance [178]. Farmers are in some areas more prone to drink more alcohol than the average population whereas in Sweden farmers drink less alcohol than average [179]. Swine dust is already known to reduce TLR2 in farmers and it could be further investigated if this is synergistically reduced in farmers with high alcohol and/or cigarette consumption.

In paper III we found that palladium particles was moved into one spot by the beating cilia. Futrure studies could investigate if other substances interact with the model differently. Previously, cilia beating frequency was reduced *in vitro* using bovine ciliated cells exposed to dissolved organic swine dust [180]. Would swine dust exposure change the cilia beating frequency or direction if using the 3D model?

Primary macrophages have to date been difficult to combine with 3D model. If this would be possible in the future, the interesting results of Ji *et al*, 2018 regarding macrophage anti-inflammatory M2 polarization after exposure to diesel exhaust particles could be further explored [49]. Alveolar macrophages from farmers expressed more anti-inflammatory *IL13* upon budesonide treatment than before treatment. Further research could investigate if freshly

prepared macrophages from different human populations could be used in the 3D model and if other ultrafine and ambient particles also would result in M2 polarisation.

CONCLUSIONS

Paper I:

- Organic dust including endotoxins is present in swine building environment.
- Installing particle separators (cyclones) in swine buildings reduced mainly the fine (0.3-0.5 μm) particles.
- The adverse acute symptoms including headache and increased body temperature in healthy volunteers exposed to organic swine dust were reduced in the particle separated swine building environment compared to the conventional swine building environment.
- The particles separated environment reduced the upper respiratory tract pro-inflammatory responses (IL-6 and CXCL8) in healhy volunteers compared to the conventional swine building.
- The effectivness of cyclones could possible be improved if used when particles numbers are high(er) i.e. during winter months.

Paper II:

- Swine farmers chronically exposed to organic dust who inhale glucocorticosteroid for two weeks increased the release of soluble TLR2 in the airways.
- Systemic effects after budesonide treatment in the farmers include increased number of circulating leucocytes and TLR4 expression on lymphocytes, and decreased cytotoxic T-cell production of IL-13 and IL-4.
- In alveolar macrophages from a smaller subset of farmers mRNA *TLR2* expression increased and *CXCL8* decreased after *ex vivo* co-stimulation with LPS/peptidoglycan/TNF-α and budesonide.
- The mRNA expression of *CD14*, *IL-13* and *GPx* in alveolar macrophages increased after the *in vivo* steroid treatment of swine farmers.
- The study showed that inhalation of a glucocorticosteroid strengthens the immune defence pathways in subjects with occupational chronic exposure to organic dust.

Paper III:

- We established a viable and robust *in vitro* bronchial mucosa co-culture model using human primary bronchial cell and fibroblast cell line showing *in vivo* cell characteristics.
- We modulated the model by stimulation with IL-13 to allow differentiation into a more chronic bronchitis-like model.
- These models were successfully combined with the advanced aerosol exposure system PreciseInhale™ and the *in vitro* module Xpose*ALI*® and exposed to palladium nanoparticles.
- Palladium nanoparticles induced inflammatory responses in the 3D model.
- The 3D model could be further refined and developed for future use in replacing acute and chronic animal tests studying local effects.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Sällan andas vi ren luft. Är den inte naturligt förorenad med pollen, sand, jord eller salt så har människan förorenat den med avgaser i form av gaser och partiklar från trafik och industri. Ett av Sverige miljömål 'Frisk luft' definieras som "Luften ska vara så ren att människors hälsa samt djur, växter och kulturvärden inte skadas."

Hur vet vi att dessa värden inte skadas? För att förstå det krävs kunskap om ämnens inneboende egenskaper och hur de påverkar sin omgivning.

Många arbetsplatser har förorenad luft och speciellt utsatta är svinskötare. Damm som bildas i svinstallar i samband med grishållning innehåller många olika beståndsdelar, bland annat rester från foder, hud, urin och avföring. Detta organiska svindamm är rikt på mikro-organismer inklusive bakterier och kan påverka både svinens och svinskötarnas hälsa. Någonting i dammet gör att kroppens immunförsvar påverkas och hur kroppen reagerar beror på hur ofta du exponeras för svindammet. En person som aldrig eller sällan vistats i svinhus kommer att inom 24 timmar efter påbörjad exponering få influensaliknande symptom som går över efter ett par dygn. En svinskötare som dagligen arbetar in svinhusmiljö har istället utvecklat tolerans mot miljön genom ett förändrat immunförsvar och får avsevärt mildare symptom efter svinhusvistelse. Trots detta är hosta vanligt bland svinskötare och de har högre risk att utveckla kronisk bronkit och kronisk obstruktiv lungsjukdom (KOL) än övriga befolkningen.

Denna avhandling syftar till att öka kunskapen kring hur man med en teknisk uppfinning kan minska exponering för organisk damm i svinhus för att förbättra svinskötares hälsa, om ett välanvänt läkemedel kan påverka förändringarna på immunförsvaret som uppkommer hos svinbönder efter långvarigt arbete i svinstallar samt ta fram en ny förfinad laboratoriemetod för att undersöka små luftburna partiklars toxicitet vilket skulle undvika framtida försök på djur och människa.

I det första delarbetet installerades cykloner i ett av två identiska svinhus, i vilka bägge sedan friska, frivilliga vistades under 3 timmar. Före och efter svinhusbesöken, som skedde med några veckors mellanrum, undersöktes försökspersonerna. De fyllde i en symptomenkät samt genomförde spirometri, nässköljning och blodprovstagning. Dessutom karakteriserades exponeringen och partiklarna i svinhusluften. Cyklonerna resulterade i färre antal partiklar, speciellt de små i storleken 0,3-0,5 µm jämfört med det konventionella stallet utan installerade cykloner. Efter att försökspersonerna vistats i stallet med installerade cykloner upplevdes mindre huvudvärk och deras kroppstemperatur ökade inte lika mycket som efter att de vistats i det konventionella stallet. Försökspersonernas immunförsvar i både blod och nässköljvätska reagerade olika beroende på vilket svinhus de exponerats för. Generellt ökade antalet "bakteriereceptorer" (TLR2 och TLR4) på blodmonocyter (en typ av vita blodkroppar som är viktiga för det medfödda immunförsvaret) och små signalmolekyler, med uppgift att aktivera inflammation, i nässköljvätska.

I det andra delarbetet rekryterades 15 friska, icke-rökande svinskötare som under två veckor antingen behandlades med inhalationssteroid (budesonid, en vanlig astmamedicin) eller placebo samtidigt som de fortsatte arbeta i svinhus och därmed exponerades för svinhusdamm. Före och efter behandling fick svinskötarna fylla i symptomenkät och genomgå blodprovstagning, spirometri samt inducera sputum (en metod för att hosta upp slem från nedre luftvägarna). Efter budesonid-behandling stimulerades svinskötarnas immunförsvar genom att antalet vita blodkroppar och ”bakteriereceptorerna” (TLR4 på blodlymfocyter och lösliga TLR2 i slemmet från nedre luftvägarna) ökade samt anti-inflammatoriska signalmolekyler i blodets T-lymfocyter minskade.

Nästan hälften av svinskötarna genomgick mer djuplodande undersökningar före och efter budesonid-behandling i form av bronkoalveolärt lavage vilket utfördes under bedövning på sjukhus. Genom att skölja lungorna med koksalt kan celler (alveolära makrofager) samlas in för vidare stimulering utanför kroppen, så kallade *ex vivo*-försök. I laboratorium utsätts makrofagerna med olika inflammationshöjande stimuli med eller utan budesonid. Stimulering med budesonid påvisar att svinböndernas immunförsvar, som annars är neddreglerat på grund av kronisk exponering i svinhus, nu förbättras till att mer likna nivåer som återfinns hos befolkningen i övrigt. Bland annat ökar tillverkningen av ”bakteriereceptorn” TLR2 i makrofagerna.

I det tredje delarbetet tillverkade vi en tredimensionell cellmodell där vi sam-odlade olika typer av human lungceller för att efterlikna mänsklig lungvävnad. Det vanliga vid cellodling är att odla celler i cellmedium. Här ville vi simulera lungvävnad där cellerna utsätts för omgivande luft vid inandning. Cellmodellen bestod av bindvävsceller och bronkiella epitelceller som, under odling med luft på ovansidan och cellmedia endast under cellerna, ombildades till olika typer av celler som normalt återfinns i luftrören: flimmerhårsceller, slemutsöndrande bägarceller och stamceller som klubbceller och basalceller. Vi kunde också stimulera slemtillverkning och på så vis efterlikna en lungmodell med kronisk bronkit. För att undersöka hur lungmodellerna reagerade på luftföroreningar (i detta fall palladium-nanopartiklar, förekommande i avgaser från motorfordon) kombinerades modellen med ett nytt exponeringssystem: PreciseInhale™ & Xpose*ALI*®. Denna kombination gör att man kan exponera celler för partiklar i luft, i stället för som brukligt med partiklar lösta i cellmedia. Denna dimension gör att inte bara cellmodellen efterliknar lungvävnad men också exponeringen liknar den som sker i en lunga via luft-blodbarriären.

Denna avhandling visar att det går att minska svindammspåverkan hos människa genom att antingen minska exponeringen genom tekniska åtgärder eller med steroidbehandling. Våra framtagna humana lungmodeller och exponeringssystem kan tillsammans användas för att vidare studera toxiska och immunologiska effekter av partiklar i luft exempelvis i svinhus.

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REFERENCES

1. WHO. *Health effects of particulate matter*. 2013; Available from: http://www.euro.who.int/__data/assets/pdf_file/0006/189051/Health-effects-of-particulate-matter-final-Eng.pdf

2. Oberdorster, G., V. Stone, and K. Donaldson, *Toxicology of nanoparticles: A historical perspective.* Nanotoxicology, 2007. **1**(1): p. 2-25.

3. Monteiller, C., et al., *The pro-inflammatory effects of low-toxicity low-solubility particles, nanoparticles and fine particles, on epithelial cells in vitro: the role of surface area.* Occup Environ Med, 2007. **64**(9): p. 609-15.

4. Brook, R.D., *Cardiovascular effects of air pollution.* Clin Sci (Lond), 2008. **115**(6): p. 175-87.

5. Miyata, R. and S.F. van Eeden, *The innate and adaptive immune response induced by alveolar macrophages exposed to ambient particulate matter.* Toxicol Appl Pharmacol, 2011. **257**(2): p. 209-26.

6. Donaldson, K., et al., *Role of inflammation in cardiopulmonary health effects of PM.* Toxicol Appl Pharmacol, 2005. **207**(2 Suppl): p. 483-8.

7. Hogg, J.C. and S. van Eeden, *Pulmonary and systemic response to atmospheric pollution.* Respirology, 2009. **14**(3): p. 336-46.

8. Dominici, F., et al., *Fine particulate air pollution and hospital admission for cardiovascular and respiratory diseases.* JAMA, 2006. **295**(10): p. 1127-1134.

9. Hiraiwa, K. and S.F. van Eeden, *Contribution of lung macrophages to the inflammatory responses induced by exposure to air pollutants.* Mediators Inflamm, 2013. **2013**: p. 619523.

10. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, I., *Outdoor Air Pollution.* IARC Monogr Eval Carcinog Risks Hum, 2016. **109**: p. 9-444.

11. Arbetsmiljöverket. *Hygieniska gränsvärden 2018:1*. Arbetsmiljöverkets författningssamling 2018; Available from: https://www.av.se/globalassets/filer/publikationer/foreskrifter/hygieniska-gransvarden-afs-2018-1.pdf

12. Eduard, W., et al., *Bioaerosol exposure assessment in the workplace: the past, present and recent advances.* J Environ Monit, 2012. **14**(2): p. 334-9.

13. Palmberg, L., et al., *Partial protection by respirators on airways responses following exposure in a swine house.* Am J Ind Med, 2004. **46**(4): p. 363-70.

14. Basinas, I., et al., *Exposure to Inhalable Dust and Endotoxin Among Danish Pig Farmers Affected by Work Tasks and Stable Characteristics.* Annals of Occupational Hygiene, 2013. **57**(8): p. 1005-1019.

15. Hiel, D., et al., *Evaluation of respiratory effects related to high-pressure cleaning in a piggery with and without robot pre-cleaning.* Scand J Work Environ Health, 2009. **35**(5): p. 376-83.

16. Beutler, B. and E.T. Rietschel, *Innate immune sensing and its roots: the story of endotoxin.* Nat Rev Immunol, 2003. **3**(2): p. 169-76.

17. Radon, K., *The two sides of the "endotoxin coin".* Occupational and Environmental Medicine, 2006. **63**(1): p. 73-78.

18. Netherlands., H.C.o.t., *Endotoxins. Health-based recommended occupational exposure limit.* Vol. Publication no. 2010/04OSH. 2010, The Hague: Health Council of the Netherlands.

19. Basinas, I., et al., *Exposure to inhalable dust and endotoxin among Danish livestock farmers: results from the SUS cohort study.* J Environ Monit, 2012. **14**(2): p. 604-14.

20. Basinas, I., et al., *Exposure-Affecting Factors of Dairy Farmers' Exposure to Inhalable Dust and Endotoxin.* Annals of Occupational Hygiene, 2014. **58**(6): p. 707-723.

21. Basinas, I., et al., *A comprehensive review of levels and determinants of personal exposure to dust and endotoxin in livestock farming.* J Expo Sci Environ Epidemiol, 2015. **25**(2): p. 123-37.

22. Poole, J.A., et al., *Toll-like receptor 2 regulates organic dust-induced airway inflammation.* Am J Respir Cell Mol Biol, 2011. **45**(4): p. 711-9.

23. Larsson, B.M., et al., *Gram positive bacteria induce IL-6 and IL-8 production in human alveolar macrophages and epithelial cells.* Inflammation, 1999. **23**(3): p. 217-30.

24. Sahlander, K., K. Larsson, and L. Palmberg, *Altered innate immune response in farmers and smokers.* Innate Immun, 2010. **16**(1): p. 27-38.

25. Nakayama, K., et al., *Involvement of IRAK-M in peptidoglycan-induced tolerance in macrophages.* J Biol Chem, 2004. **279**(8): p. 6629-34.

26. Nahid, M.A., et al., *Regulation of TLR2-mediated tolerance and cross-tolerance through IRAK4 modulation by miR-132 and miR-212.* J Immunol, 2013. **190**(3): p. 1250-63.

27. Hamon, L., Y. Andres, and E. Dumont, *Aerial Pollutants in Swine Buildings: A Review of Their Characterization and Methods to Reduce Them.* Environmental Science & Technology, 2012. **46**(22): p. 12287-12301.

28. Pedersen, S., et al., *Dust in Pig Buildings.* Journal of Agricultural Safety and Health, 2000. **6**(4): p. 261.

29. Senthilselvan, A., et al., *Positive human health effects of dust suppression with canola oil in swine barns.* Am J Respir Crit Care Med, 1997. **156**(2 Pt 1): p. 410-7.

30. Siggers, J.L., et al., *Size Distribution of Particulate and Associated Endotoxin and Bacteria in Traditional Swine Barn Rooms and Rooms Sprinkled With Oil.* Journal of Agromedicine, 2011. **16**(4): p. 271-279.

31. Banhazi, T.M., et al., *Identification of risk factors for sub-optimal housing conditions in Australian piggeries: Part 2. Airborne pollutants.* J Agric Saf Health, 2008. **14**(1): p. 21-39.

32. Dosman, J.A., et al., *Positive human health effects of wearing a respirator in a swine barn.* Chest, 2000. **118**(3): p. 852-60.

33. Bonlokke, J.H., et al., *Work-Related Health Effects in Swine Building Workers After Respiratory Protection Use.* Journal of Occupational and Environmental Medicine, 2012. **54**(9): p. 1126-1132.

34. Sundblad, B.M., et al., *Effect of respirators equipped with particle or particle-and-gas filters during exposure in a pig confinement building.* Scand J Work Environ Health, 2006. **32**(2): p. 145-53.

35. Larsson, B.M., et al., *Airways inflammation after exposure in a swine confinement building during cleaning procedure.* Am J Ind Med, 2002. **41**(4): p. 250-8.

36. Basinas, I., et al., *Feedback on Measured Dust Concentrations Reduces Exposure Levels Among Farmers.* Ann Occup Hyg, 2016. **60**(7): p. 812-24.

37. Berube, K., et al., *Human primary bronchial lung cell constructs: the new respiratory models.* Toxicology, 2010. **278**(3): p. 311-8.

38. Schamberger, A.C., et al., *Cigarette smoke alters primary human bronchial epithelial cell differentiation at the air-liquid interface.* Sci Rep, 2015. **5**: p. 8163.

39. Laucho-Contreras, M.E., et al., *Club Cell Protein 16 (CC16) Augmentation: A Potential Disease-modifying Approach for Chronic Obstructive Pulmonary Disease (COPD).* Expert Opin Ther Targets, 2016. **20**(7): p. 869-83.

40. Lodish H, B.A., Zipursky SL, et al., *Molecular Cell Biology*. 2000, W. H. Freeman: New York.

41. Rock, J.R., S.H. Randell, and B.L.M. Hogan, *Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling.* Disease Models & Mechanisms, 2010. **3**(9-10): p. 545-556.

42. Rose, M.C., T.J. Nickola, and J.A. Voynow, *Airway mucus obstruction: mucin glycoproteins, MUC gene regulation and goblet cell hyperplasia.* Am J Respir Cell Mol Biol, 2001. **25**(5): p. 533-7.

43. Atherton, H.C., G. Jones, and H. Danahay, *IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation.* Am J Physiol Lung Cell Mol Physiol, 2003. **285**(3): p. L730-9.

44. Kikuchi, T., et al., *Differentiation-dependent responsiveness of bronchial epithelial cells to IL-4/13 stimulation.* Am J Physiol Lung Cell Mol Physiol, 2004. **287**(1): p. L119-26.

45. Ji, J., et al., *Development of Combining of Human Bronchial Mucosa Models with XposeALI (R) for Exposure of Air Pollution Nanoparticles.* Plos One, 2017. **12**(1).

46. Twigg, H.L., 3rd, *Macrophages in innate and acquired immunity.* Semin Respir Crit Care Med, 2004. **25**(1): p. 21-31.

47. Byrne, A.J., et al., *Pulmonary macrophages: key players in the innate defence of the airways.* Thorax, 2015. **70**(12): p. 1189-96.

48. Wang, N., H. Liang, and K. Zen, *Molecular Mechanisms That Influence the Macrophage M1–M2 Polarization Balance.* Frontiers in Immunology, 2014. **5**: p. 614.

49. Ji, J., et al., *Multi-cellular human bronchial models exposed to diesel exhaust particles: assessment of inflammation, oxidative stress and macrophage polarization.* Particle and Fibre Toxicology, 2018. **15**.

50. Barnes, P.J., *Inflammatory mechanisms in patients with chronic obstructive pulmonary disease.* J Allergy Clin Immunol, 2016. **138**(1): p. 16-27.

51. Caramalho, I., et al., *Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide.* J Exp Med, 2003. **197**(4): p. 403-11.

52. Komai-Koma, M., et al., *TLR2 is expressed on activated T cells as a costimulatory receptor.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 3029-34.

53. Liu, G., L. Zhang, and Y. Zhao, *Modulation of immune responses through direct activation of Toll-like receptors to T cells.* Clin Exp Immunol, 2010. **160**(2): p. 168-75.

54. Summers, C., et al., *Neutrophil kinetics in health and disease.* Trends Immunol, 2010. **31**(8): p. 318-24.

55. Borregaard, N., *Neutrophils, from marrow to microbes.* Immunity, 2010. **33**(5): p. 657-70.

56. Dahlgren, C. and A. Karlsson, *Respiratory burst in human neutrophils.* J Immunol Methods, 1999. **232**(1-2): p. 3-14.

57. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.

58. O'Neill, L.A., D. Golenbock, and A.G. Bowie, *The history of Toll-like receptors - redefining innate immunity.* Nat Rev Immunol, 2013. **13**(6): p. 453-60.

59. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity.* International Immunology, 2005. **17**(1): p. 1-14.

60. Becker, S., M.J. Fenton, and J.M. Soukup, *Involvement of microbial components and toll-like receptors 2 and 4 in cytokine responses to air pollution particles.* Am J Respir Cell Mol Biol, 2002. **27**(5): p. 611-8.

61. Takeuchi, O., et al., *Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components.* Immunity, 1999. **11**(4): p. 443-51.

62. Janeway, C.A. and R. Medzhitov, *Innate immune recognition.* Annual Review of Immunology, 2002. **20**: p. 197-216.

63. Kaisho, T. and S. Akira, *Toll-like receptor function and signaling.* J Allergy Clin Immunol, 2006. **117**(5): p. 979-87; quiz 988.

64. Ten Oever, J., et al., *The discriminative capacity of soluble Toll-like receptor (sTLR)2 and sTLR4 in inflammatory diseases.* BMC Immunol, 2014. **15**: p. 55.

65. von Scheele, I., et al., *Toll-like receptor expression in smokers with and without COPD.* Respir Med, 2011. **105**(8): p. 1222-30.

66. Droemann, D., et al., *Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients.* Respir Res, 2005. **6**: p. 68.

67. Pons, J., et al., *Expression of Toll-like receptor 2 is up-regulated in monocytes from patients with chronic obstructive pulmonary disease.* Respiratory Research, 2006. **7**.

68. Southworth, T., et al., *IFN-gamma synergistically enhances LPS signalling in alveolar macrophages from COPD patients and controls by corticosteroid-resistant STAT1 activation.* Br J Pharmacol, 2012. **166**(7): p. 2070-83.

69. Ji, J., et al., *Effects of budesonide on toll-like receptor expression in alveolar macrophages from smokers with and without COPD.* International Journal of Chronic Obstructive Pulmonary Disease, 2016. **11**: p. 1035-1043.

70. Zunt, S.L., et al., *Soluble forms of Toll-like receptor 4 are present in human saliva and modulate tumour necrosis factor-alpha secretion by macrophage-like cells.* Clin Exp Immunol, 2009. **156**(2): p. 285-93.

71. Wright, S.D., *CD14 and innate recognition of bacteria.* J Immunol, 1995. **155**(1): p. 6-8.

72. Pugin, J., et al., *Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14.* Proc Natl Acad Sci U S A, 1993. **90**(7): p. 2744-8.

73. Kitchens, R.L. and P.A. Thompson, *Modulatory effects of sCD14 and LBP on LPS-host cell interactions.* J Endotoxin Res, 2005. **11**(4): p. 225-9.

74. Anas, A., T. van der Poll, and A.F. de Vos, *Role of CD14 in lung inflammation and infection.* Crit Care, 2010. **14**(2): p. 209.

75. Mukaida, N., *Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases.* Am J Physiol Lung Cell Mol Physiol, 2003. **284**(4): p. L566-77.

76. Tanaka, T., M. Narazaki, and T. Kishimoto, *IL-6 in inflammation, immunity, and disease.* Cold Spring Harb Perspect Biol, 2014. **6**(10): p. a016295.

77. Rincon, M. and C.G. Irvin, *Role of IL-6 in asthma and other inflammatory pulmonary diseases.* Int J Biol Sci, 2012. **8**(9): p. 1281-90.

78. Sahlander, K., K. Larsson, and L. Palmberg, *Increased serum levels of soluble ST2 in birch pollen atopics and individuals working in laboratory animal facilities.* J Occup Environ Med, 2010. **52**(2): p. 214-8.

79. Tang, Y., et al., *The Role of the Serum IL-33/sST2 Axis and Inflammatory Cytokines in Chronic Obstructive Pulmonary Disease.* Journal of Interferon and Cytokine Research, 2014. **34**(3): p. 162-168.

80. Pascual-Figal, D.A. and J.L. Januzzi, *The biology of ST2: the International ST2 Consensus Panel.* Am J Cardiol, 2015. **115**(7 Suppl): p. 3B-7B.

81. Sahlander, K., K. Larsson, and L. Palmberg, *Daily exposure to dust alters innate immunity.* PLoS One, 2012. **7**(2): p. e31646.

82. Zhu, Z., et al., *Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production.* J Clin Invest, 1999. **103**(6): p. 779-88.

83. Brightling, C.E., S. Saha, and F. Hollins, *Interleukin-13: prospects for new treatments.* Clin Exp Allergy, 2010. **40**(1): p. 42-9.

84. Miotto, D., et al., *Interleukin-13 and -4 expression in the central airways of smokers with chronic bronchitis.* Eur Respir J, 2003. **22**(4): p. 602-8.

85. Chakrabarti, S. and K.D. Patel, *Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology.* Exp Lung Res, 2005. **31**(6): p. 599-621.

86. Hoshino, M., et al., *Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and*

tissue inhibitor of metalloproteinase-1 expression in asthma. J Allergy Clin Immunol, 1999. **104**(2 Pt 1): p. 356-63.

87. Linder, R., et al., *Serum metalloproteinase-9 is related to COPD severity and symptoms - cross-sectional data from a population based cohort-study.* Respir Res, 2015. **16**: p. 28.

88. Nelson, S. and C.M. Mason, *The inflammatory response in chronic bronchitis.* Semin Respir Crit Care Med, 2000. **21**(2): p. 79-86.

89. Heath, J.M. and R. Mongia, *Chronic bronchitis: primary care management.* Am Fam Physician, 1998. **57**(10): p. 2365-72, 2376-8.

90. Lundbäck, B., et al., *Not 15 But 50% of smokers develop COPD?—Report from the Obstructive Lung Disease in Northern Sweden Studies.* Respiratory Medicine, 2003. **97**(2): p. 115-122.

91. Fuller-Thomson, E., R.S. Chisholm, and S. Brennenstuhl, *COPD in a Population-Based Sample of Never-Smokers: Interactions among Sex, Gender, and Race.* Int J Chronic Dis, 2016. **2016**: p. 5862026.

92. Larsson, K., *Aspects on pathophysiological mechanisms in COPD.* J Intern Med, 2007. **262**(3): p. 311-40.

93. GOLD, *Global strategy for the diagnosis, management, and prevention of chronich obstructive pulmonary disease*. 2018.

94. GINA, *Global strategy for asthma prevention and management*. 2018.

95. Holgate, S.T., *Pathogenesis of asthma.* Clin Exp Allergy, 2008. **38**(6): p. 872-97.

96. Sperber, J., et al., *Lung protective ventilation induces immunotolerance and nitric oxide metabolites in porcine experimental postoperative sepsis.* PLoS One, 2013. **8**(12): p. e83182.

97. Rask-Andersen, A., *Organic Dust Toxic Syndrome among Farmers.* British Journal of Industrial Medicine, 1989. **46**(4): p. 233-238.

98. Larsson, B.M., et al., *Effect of exposure to swine dust on levels of IL-8 in airway lavage fluid.* Thorax, 1997. **52**(7): p. 638-42.

99. Dosman, J.A., et al., *Respiratory response to endotoxin and dust predicts evidence of inflammatory response in volunteers in a swine barn.* Am J Ind Med, 2006. **49**(9): p. 761-6.

100. Palmberg, L., et al., *Airway responses of healthy farmers and nonfarmers to exposure in a swine confinement building.* Scand J Work Environ Health, 2002. **28**(4): p. 256-63.

101. Sundblad, B.M., et al., *Repeated exposure to organic material alters inflammatory and physiological airway responses.* Eur Respir J, 2009. **34**(1): p. 80-8.

102. Hoffmann, H.J., et al., *A single exposure to organic dust of non-naive non-exposed volunteers induces long-lasting symptoms of endotoxin tolerance.* Int Arch Allergy Immunol, 2005. **138**(2): p. 121-6.

103. Rask-Andersen, A., P. Malmberg, and M. Lundholm, *Endotoxin levels in farming: absence of symptoms despite high exposure levels.* Br J Ind Med, 1989. **46**(6): p. 412-6.

104. Zhiping, W., et al., *Exposure to bacteria in swine-house dust and acute inflammatory reactions in humans.* Am J Respir Crit Care Med, 1996. **154**(5): p. 1261-6.

105. Hedelin, A.S., et al., *Comparing human respiratory adverse effects after acute exposure to particulate matter in conventional and particle-reduced swine building environments.* Occupational and Environmental Medicine, 2016. **73**(10): p. 648-655.

106. Poole, J.A., et al., *Muramic acid, endotoxin, 3-hydroxy fatty acids, and ergosterol content explain monocyte and epithelial cell inflammatory responses to agricultural dusts.* J Toxicol Environ Health A, 2010. **73**(10): p. 684-700.

107. Chen, L.C. and M. Lippmann, *Inhalation toxicology methods: the generation and characterization of exposure atmospheres and inhalational exposures.* Curr Protoc Toxicol, 2015. **63**: p. 24 4 1-23.

108. Charavaryamath, C., et al., *Multiple exposures to swine barn air induce lung inflammation and airway hyper-responsiveness.* Respir Res, 2005. **6**: p. 50.

109. Poole, J.A., et al., *Intranasal organic dust exposure-induced airway adaptation response marked by persistent lung inflammation and pathology in mice.* Am J Physiol Lung Cell Mol Physiol, 2009. **296**(6): p. L1085-95.

110. Clift, M.J., P. Gehr, and B. Rothen-Rutishauser, *Nanotoxicology: a perspective and discussion of whether or not in vitro testing is a valid alternative.* Arch Toxicol, 2010.

111. Upadhyay, S. and L. Palmberg, *Air-Liquid Interface: Relevant In Vitro Models for Investigating Air Pollutant-Induced Pulmonary Toxicity.* Toxicological Sciences, 2018. **164**(1): p. 21-30.

112. Svensson, C.R., et al., *Validation of an air-liquid interface toxicological set-up using Cu, Pd, and Ag well-characterized nanostructured aggregates and spheres.* Journal of Nanoparticle Research, 2016. **18**(4).

113. Barnes, P.J., *Mechanisms and resistance in glucocorticoid control of inflammation.* J Steroid Biochem Mol Biol, 2010. **120**(2-3): p. 76-85.

114. Kadmiel, M. and J.A. Cidlowski, *Glucocorticoid receptor signaling in health and disease.* Trends Pharmacol Sci, 2013. **34**(9): p. 518-30.

115. Bel, E.H. and A. Ten Brinke, *New Anti-Eosinophil Drugs for Asthma and COPD: Targeting the Trait!* Chest, 2017. **152**(6): p. 1276-1282.

116. Durham, A., I.M. Adcock, and O. Tliba, *Steroid resistance in severe asthma: current mechanisms and future treatment.* Curr Pharm Des, 2011. **17**(7): p. 674-84.

117. Busillo, J.M. and J.A. Cidlowski, *The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore.* Trends Endocrinol Metab, 2013. **24**(3): p. 109-19.

118. Cruz-Topete, D. and J.A. Cidlowski, *One hormone, two actions: anti- and pro-inflammatory effects of glucocorticoids.* Neuroimmunomodulation, 2015. **22**(1-2): p. 20-32.

119. Pace, E., et al., *Budesonide increases TLR4 and TLR2 expression in Treg lymphocytes of allergic asthmatics.* Pulm Pharmacol Ther, 2015. **32**: p. 93-100.

120. Spengler, C.M. and S.A. Shea, *Endogenous circadian rhythm of pulmonary function in healthy humans.* Am J Respir Crit Care Med, 2000. **162**(3 Pt 1): p. 1038-46.

121. *ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005.* Am J Respir Crit Care Med, 2005. **171**(8): p. 912-30.

122. Durrington, H.J., et al., *The circadian clock and asthma.* Thorax, 2014. **69**(1): p. 90-2.

123. Labrecque, N. and N. Cermakian, *Circadian Clocks in the Immune System.* J Biol Rhythms, 2015. **30**(4): p. 277-90.

124. Taylor, D.R., et al., *Exhaled nitric oxide measurements: clinical application and interpretation.* Thorax, 2006. **61**(9): p. 817-27.

125. Barnes, P.J., et al., *Exhaled nitric oxide in pulmonary diseases: a comprehensive review.* Chest, 2010. **138**(3): p. 682-92.

126. Zetterquist, W., et al., *Salivary contribution to exhaled nitric oxide.* Eur Respir J, 1999. **13**(2): p. 327-33.

127. Paggiaro, P.L., et al., *Sputum induction.* Eur Respir J Suppl, 2002. **37**: p. 3s-8s.

128. Grootendorst, D.C., et al., *Comparison of inflammatory cell counts in asthma: Induced sputum vs bronchoalveolar lavage and bronchial biopsies.* Clinical and Experimental Allergy, 1997. **27**(7): p. 769-779.

129. Olsen, H.H., et al., *Bronchoalveolar Lavage Results Are Independent of Season, Age, Gender and Collection Site.* Plos One, 2012. **7**(8).

130. Wilkinson, K.E., et al., *Solution-Engineered Palladium Nanoparticles: Model for Health Effect Studies of Automotive Particulate Pollution.* Acs Nano, 2011. **5**(7): p. 5312-5324.

131. Fischer, A.H., et al., *Hematoxylin and eosin staining of tissue and cell sections.* CSH Protoc, 2008. **2008**: p. pdb prot4986.

132. Hauber, H.P. and P. Zabel, *PAS staining of bronchoalveolar lavage cells for differential diagnosis of interstitial lung disease.* Diagn Pathol, 2009. **4**: p. 13.

133. Srinivasan, B., et al., *TEER measurement techniques for in vitro barrier model systems.* J Lab Autom, 2015. **20**(2): p. 107-26.

134. Schuerwegh, A.J., et al., *Evaluation of monensin and brefeldin A for flow cytometric determination of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha in monocytes.* Cytometry, 2001. **46**(3): p. 172-6.

135. Thorne, P.S., et al., *Evaluation of the Limulus amebocyte lysate and recombinant factor C assays for assessment of airborne endotoxin.* Appl Environ Microbiol, 2010. **76**(15): p. 4988-95.

136. Reynolds, S.J., et al., *Interlaboratory evaluation of endotoxin analyses in agricultural dusts--comparison of LAL assay and mass spectrometry.* J Environ Monit, 2005. **7**(12): p. 1371-7.

137. Duquenne, P., G. Marchand, and C. Duchaine, *Measurement of Endotoxins in Bioaerosols at Workplace: A Critical Review of Literature and a Standardization Issue.* Annals of Occupational Hygiene, 2013. **57**(2): p. 137-172.

138. Chan, A.T., *Indoor-outdoor relationships of particulate matter and nitrogen oxides under different outdoor meteorological conditions.* Atmospheric Environment, 2002. **36**(9): p. 1543-1551.

139. Hussein, T., et al., *Particle size characterization and the indoor-to-outdoor relationship of atmospheric aerosols in Helsinki.* Scand J Work Environ Health, 2004. **30 Suppl 2**: p. 54-62.

140. Preller, L., et al., *Determinants of dust and endotoxin exposure of pig farmers: development of a control strategy using empirical modelling.* Ann Occup Hyg, 1995. **39**(5): p. 545-57.

141. Bonlokke, J.H., et al., *Seasonal Variations in Work-Related Health Effects in Swine Farm Workers.* Annals of Agricultural and Environmental Medicine, 2009. **16**(1): p. 43-52.

142. Sundblad, B.M., et al., *Exhaled nitric oxide and bronchial responsiveness in healthy subjects exposed to organic dust.* Eur Respir J, 2002. **20**(2): p. 426-31.

143. Evans, S.S., E.A. Repasky, and D.T. Fisher, *Fever and the thermal regulation of immunity: the immune system feels the heat.* Nat Rev Immunol, 2015. **15**(6): p. 335-49.

144. Vgontzas, A.N., et al., *IL-6 and its circadian secretion in humans.* Neuroimmunomodulation, 2005. **12**(3): p. 131-40.

145. Wang, Z.P., et al., *Time course of interleukin-6 and tumor necrosis factor-alpha increase in serum following inhalation of swine dust.* American Journal of Respiratory and Critical Care Medicine, 1996. **153**(1): p. 147-152.

146. von Scheele, I., K. Larsson, and L. Palmberg, *Budesonide enhances Toll-like receptor 2 expression in activated bronchial epithelial cells.* Inhal Toxicol, 2010. **22**(6): p. 493-9.

147. Barczyk, A., et al., *Cytokine production by bronchoalveolar lavage T lymphocytes in chronic obstructive pulmonary disease.* J Allergy Clin Immunol, 2006. **117**(6): p. 1484-92.

148. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. **8**(12): p. 958-69.

149. Palmberg, L., et al., *Induction of IL-8 production in human alveolar macrophages and human bronchial epithelial cells in vitro by swine dust.* Thorax, 1998. **53**(4): p. 260-4.

150. Ek, A., et al., *Fluticasone and budesonide inhibit cytokine release in human lung epithelial cells and alveolar macrophages.* Allergy, 1999. **54**(7): p. 691-9.

151. LeVan, T.D., et al., *Polymorphisms in the CD14 gene associated Ewith pulmonary function in farmers.* American Journal of Respiratory and Critical Care Medicine, 2005. **171**(7): p. 773-779.

152. de Jong, P.M., et al., *Ciliogenesis in human bronchial epithelial cells cultured at the air-liquid interface.* Am J Respir Cell Mol Biol, 1994. **10**(3): p. 271-7.

153. Choe, M.M., A.A. Tomei, and M.A. Swartz, *Physiological 3D tissue model of the airway wall and mucosa.* Nat Protoc, 2006. **1**(1): p. 357-62.

154. Unfried, K., et al., *Cellular responses to nanoparticles: Target structures and mechanisms.* Nanotoxicology, 2007. **1**(1): p. 52-71.

155. Iversen, T.G., T. Skotland, and K. Sandvig, *Endocytosis and intracellular transport of nanoparticles: Present knowledge and need for future studies.* Nano Today, 2011. **6**(2): p. 176-185.

156. Docter, D., et al., *The nanoparticle biomolecule corona: lessons learned - challenge accepted?* Chem Soc Rev, 2015. **44**(17): p. 6094-121.

157. Atkinson, J.J. and R.M. Senior, *Matrix metalloproteinase-9 in lung remodeling.* Am J Respir Cell Mol Biol, 2003. **28**(1): p. 12-24.

158. Erickson, H.P., *Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy.* Biol Proced Online, 2009. **11**: p. 32-51.

159. McClendon, C.J., C.L. Gerald, and J.T. Waterman, *Farm animal models of organic dust exposure and toxicity: insights and implications for respiratory health.* Curr Opin Allergy Clin Immunol, 2015. **15**(2): p. 137-44.

160. Eduard, W., N. Pearce, and J. Douwes, *Chronic bronchitis, COPD, and lung function in farmers: the role of biological agents.* Chest, 2009. **136**(3): p. 716-25.

161. Bolund, A.C., et al., *The effect of organic dust exposure on long-term change in lung function: a systematic review and meta-analysis.* Occup Environ Med, 2017. **74**(7): p. 531-542.

162. Radon, K., et al., *Respiratory symptoms in European animal farmers.* European Respiratory Journal, 2001. **17**(4): p. 747-754.

163. Frank, A.L., et al., *Issues of agricultural safety and health.* Annual Review of Public Health, 2004. **25**: p. 225-245.

164. Bolund, A.C.S., et al., *New-onset COPD and Decline in Lung Function Among Wood Dust-Exposed Workers: Re-analysis of a 6-year Follow-up Study.* Ann Work Expo Health, 2018.

165. Schulte, P., et al., *Occupational Risk Management of Engineered Nanoparticles.* Journal of Occupational and Environmental Hygiene, 2008. **5**(4): p. 239-249.

166. Diefenbach, H., et al., *Airborne Biological Hazards in Different PIG Fattening Systems.* International Journal of Immunopathology and Pharmacology, 2007. **20**(2_suppl): p. 45-49.

167. Geng, Q., A. Torén, and E. Salomon, *Screening the Working Environment in Outdoor Pig Systems.* Journal of Agricultural Safety and Health, 2009. **15**(3): p. 283-297.

168. Pearce, S.C., et al., *The effects of heat stress and plane of nutrition on metabolism in growing pigs.* J Anim Sci, 2013. **91**(5): p. 2108-18.

169. Schulz, J., et al., *Longitudinal Study of the Contamination of Air and of Soil Surfaces in the Vicinity of Pig Barns by Livestock-Associated Methicillin-Resistant Staphylococcus aureus.* Applied and Environmental Microbiology, 2012. **78**(16): p. 5666-5671.

170. Feld, L., et al., *Survival of LA-MRSA in Dust from Swine Farms.* Annals of Work Exposures and Health, 2018. **62**(2): p. 147-156.

171. Österberg, J., et al., *Antibiotic Resistance in Escherichia coli from Pigs in Organic and Conventional Farming in Four European Countries.* PLOS ONE, 2016. **11**(6): p. e0157049.

172. Cuny, C., L.H. Wieler, and W. Witte, *Livestock-Associated MRSA: The Impact on Humans.* Antibiotics, 2015. **4**(4): p. 521-543.

173. Hansel, T.T. and P.J. Barnes, *New drugs for exacerbations of chronic obstructive pulmonary disease.* The Lancet, 2009. **374**(9691): p. 744-755.

174. Von Essen, S. and D. Romberger, *The respiratory inflammatory response to the swine confinement building environment: the adaptation to respiratory exposures in the chronically exposed worker.* J Agric Saf Health, 2003. **9**(3): p. 185-96.

175. Bonlokke, J.H., et al., *Immunologic mechanisms in the adaptation of swine farm workers to their work environment.* Innate Immunity, 2013. **19**(4): p. 403-410.

176. Raissy, H.H., et al., *Inhaled Corticosteroids in Lung Diseases.* American Journal of Respiratory and Critical Care Medicine, 2013. **187**(8): p. 798-803.

177. Meurens, F., et al., *The pig: a model for human infectious diseases.* Trends in Microbiology, 2012. **20**(1): p. 50-57.

178. Bailey, K.L., et al., *Alcohol functionally upregulates Toll-like receptor 2 in airway epithelial cells.* Alcohol Clin Exp Res, 2009. **33**(3): p. 499-504.

179. Hemmingsson, T., et al., *Alcoholism in social classes and occupations in Sweden.* Int J Epidemiol, 1997. **26**(3): p. 584-91.

180. Wyatt, T.A., et al., *Exposure to hog barn dust alters airway epithelial ciliary beating.* European Respiratory Journal, 2008. **31**(6): p. 1249-1255.